CHARACTERISATION OF CULTURAL, BIOLOGICAL AND MOLECULAR VARIABILITY OF *PHOMA LIGULICOLA* ISOLATES ASSOCIATED WITH RAY BLIGHT DISEASE OF PYRETHRUM AND CHRYSANTHEMUM

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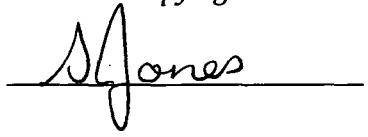

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ABSTRACT

Pyrethrum (*Tanacetum cinerariifolium*) is a perennial plant grown commercially in Tasmania, Australia for production of insecticidal pyrethrins. Tasmania produces over 30 % of the world's supply of natural pyrethrins. Ray blight disease caused by the fungal pathogen *Phoma ligulicola* var. *inoxydabilis*, is a major limiting factor of pyrethrum production in Tasmania. It causes die back of emerging stems in spring, and flower disease over summer. Most of the information regarding this pathogen is based on studies of the variety that causes ray blight on chrysanthemum, *P. ligulicola* var. *ligulicola.* The objectives of this project were to characterise the biological, cultural and genetic variability, and investigate the reproductive nature of the *P. ligulicola* var. *inoxydabilis* from pyrethrum. This study also aimed to confirm the species identity and determine the variety of *Phoma exigua* from diseased pyrethrum plants.

Assessment of morphological and cultural characteristics showed considerable variability among *P. ligulicola* isolates from pyrethrum (n = 116) and chrysanthemum (n =5). Due to the observed variability and divergence from published descriptions of the two *P. ligulicola* varieties it was not possible to reliably differentiate between the two varieties on the basis of morphological criteria alone. The most notable discrepancy was that *P. ligulicola* var. *inoxydabilis* isolates did not produce the teleomorph (*Didymella ligulicola* var. *inoxydabilis*) in culture. The two *P. ligulicola* varieties were differentiated on the basis of the presence of metabolite E and minor divergence in the ribosomal DNA region of the internal transcribed spacer (ITS) region .

Phylogenetic analysis of the translation elongation factor (EF1-α), ITS and glyceraldehyde-3-phoshpate dehydrogenase (G3PD) regions confirmed the identity of *P. ligulicola* and *P. exigua* isolates to species level, and suggested that the *P. ligulicola* var. *inoxydabilis* population from pyrethrum is largely clonal. The EF1-α and G3PD sequences did not clearly differentiate between *P.*

ligulicola varieties, the host from which they were isolated or the geographic location from which they were sourced.

Phoma ligulicola var. *inoxydabilis* isolates from pyrethrum (n = 111) were tested *in vitro* for sensitivity to the DMI fungicide difenoconazole and had a low mean EC_{50} of 0.136 µg a.i./ml. The continuous sensitivity distributions detected for the *P. ligulicola* populations indicate that resistance development for this pathosystem is likely to be a gradual process. The mean EC_{50} of four *P. exigua* isolates was 50 times greater than for *P. ligulicola* var. *inoxydabilis* isolates.

A PCR assay for determination of mating type detected the HMG motif in the five *P. ligulicola* var. *ligulicola* isolates and in one of the 116 *P. ligulicola* var. *inoxydabilis* isolates. Individual isolates of *P. ligulicola* var. *ligulicola* produced perithecia and ascospores on leaf amended agar, but none were detected for *P. ligulicola* var. *inoxydabilis*. These results indicate that the former variety was homothallic, and the latter variety was heterothallic.

The pathogenicity of 18 *P. ligulicola* and two *P. exigua* isolates to a single variety of pyrethrum was characterised in two separate experiments by inoculating detached leaves *in vitro* and glass-house grown plants. In the detached leaf assay significant differences were detected between isolates in lesion growth. There was a positive correlation between the lesion growth in the detached leaf assay and disease incidence in the greenhouse trial for both *Phoma* species. This is the first report that *P. exigua* var. *exigua* is pathogenic to commercial pyrethrum. Symptoms produced on eight week old pyrethrum plants in the greenhouse, inoculated with *P. exigua* conidial suspensions, were mid-brown leaf lesions up to 5 mm long, and occasional stem lesions.

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CHAPTER 1

INTRODUCTION AND THESIS SCOPE

Pyrethrum (*Tanacetum cinerariifolium* (Trevir.) Scholz Bip.) is a perennial daisy-like plant, grown commercially in Tasmania, Australia for extraction of insecticidal pyrethins. Pyrethrins are extracted from the flowers and used directly for pest control as well as providing the basis for production of various insecticidal products (e.g. mosquito coils and flea collars). Tasmania produces over 30% of the world's production and is the second largest producer of pyrethrum in the world. Several fungal species cause foliar and flower diseases on pyrethrum, but the most limiting is ray blight disease caused by *Phoma ligulicola* (Pethybridge et al. 2007a). Ray blight symptoms include defoliation, necrotic lesions on stems and leaves, dieback of stems and diseased flowers and buds over spring and summer (Pethybridge *et al.* 2003; Pethybridge and Wilson 1998).

The species *Phoma ligulicola* is divided into two varieties, vars. *ligulicola* and *inoxydabilis,* based on host specificity, and cultural and morphological characteristics *in vitro*, a macrochemical reaction, and sporulation in culture. *Phoma ligulicola* var. *ligulicola* is associated with ray blight disease of chrysanthemum (*Chrysanthemum morifolium*) and much of the information regarding the aetiology and lifecycle of *P. ligulicola* is based on studies of the disease on chrysanthemum. The variety associated with defoliation and ray blight of pyrethrum is *P. ligulicola* var. *inoxydabilis*. This pathogen was first reported to cause disease in pyrethrum crops in Tasmania in1995 (Pethybridge and Wilson 1998). Subsequent studies of the fungus on pyrethrum have focused on fungicide efficacy, site specific factors that influence disease development and the effect of this disease on flower and pyrethrin yields (Pethybridge *et al.* 2007a; Pethybridge *et al.* 2005a; Pethybridge *et al.* 2009; Pethybridge and Hay 2001; Pethybridge *et al.* 2008e).

Classification and identification of *Phoma* species is difficult because they are highly variable in culture and features used to separate the species frequently overlap. Additionally, geographical separation, host-pathogen interaction and exposure to various climatic environments contribute to cultural and biological variability. Correct identification of fungal isolates from diseased field plants is essential for development of appropriate disease control strategies. Information on cultural and morphological variability of *P. ligulicola* from pyrethrum is needed to ensure that new isolations from pyrethrum fields are correctly identified.

DNA-based methods, such as polymerase chain reaction (PCR) and sequencing can supplement traditional methods of fungal identification. Comparison of gene regions may also provide information on phylogenetic relationships among closely related species and genetic recombination events. Published information regarding genetic composition of *P. ligulicola* is limited to sequences of the internal transcribed spacer (ITS) regions (Pethybridge *et al.* 2004b) and phylogenic assessment of random amplified polymorphic DNA (RAPD) (Pethybridge *et al.* 2005c). Additional information is needed to supplement the existing data, and to clarify phylogenetic relationships of the two *P. ligulicola* varieties as well as related species.

The current approach to management of ray blight disease in the Tasmanian pyrethrum industry is based on the strategic use of fungicides, because no resistant pyrethrum varieties have been found. Difenoconazole is a sterol demethylation inhibitor (DMI) fungicide which has been routinely applied to pyrethrum crops in the spring fungicide program. A single crop of pyrethrum may be exposed to as many as 16 DMI fungicide applications because pyrethrum is grown for up to four years and a related fungicide, tebuconazole, is used for control of other fungal diseases. To date, disease management with fungicides has been successful (Pethybridge *et al.* 2008e). However there is a risk that with ongoing exposure to DMI fungicides, *P. ligulicola* will develop reduced sensitivity. Baseline data on fungicide sensitivity is needed so that shifts in fungicide sensitivity can be monitored.

Breeding resistance to *P. ligulicola* into pyrethrum varieties could provide the pyrethrum industry with an additional disease control method and can potentially decrease the reliance on fungicides. The Tasmanian pyrethrum plant breeding program aims to identify cultivars with reduced susceptibility to *P. ligulicola*. The susceptibility of pyrethrum to *P. ligulicola* has been assessed in the field, and also in a glasshouse study with a limited number of fungal isolates whose pathogenicity had not been characterised (Pethybridge *et al.* 2007b). Assessment of pathogenic variability in the *P. ligulicola* population is needed to ensure that isolates used to screen potential pyrethrum varieties encompass the range of pathogenicity in the field. Additionally, the methods used previously to assess cultivar susceptibility involved inoculation of plants with conidial suspensions. While this method is valid it is limited to isolates that produce sufficient quantities of spores *in vitro* and may exclude isolates that are highly pathogenic but have low sporulation capacity. An alternative method, such as inoculation with mycelia, is needed to enable testing of all *P. ligulicola* isolates.

Phoma ligulicola reproduces asexually producing splash dispersed conidia within pycnidia and sexually producing airborne ascospores within perithecia. Sexual reproduction plays an important role in the epidemiology of *P. ligulicola* through formation of airborne ascospores which aid in long range distribution of the pathogen. *Phoma ligulicola* is reported to be homothallic and both the anamorph and teleomorph stages have been described for the two varieties. Only the asexual (anamorph) stage of the *P. ligulicola* lifecycle has been isolated from infected pyrethrum plants in Tasmanian fields. The sexual stage (teleomorph), *Didymella ligulicola*, has not been found in isolations collected from the field, nor produced by Tasmanian cultures studied *in vitro.* This may suggest that the Tasmanian *P. ligulicola* isolates are heterothallic, and require two different mating types for sexual reproduction, but only one mating type is present. If the teleomorph is not present or capable of forming, disease management strategies can focus on reducing infield spread due to splash dispersal of conidia rather than on longer range dispersal of inoculum between fields.

While conducting isolations from pyrethrum with symptoms of *P. ligulicola*, isolates tentatively identified as *Phoma exigua*, were collected from diseased pyrethrum plants. *Phoma exigua* is a ubiquitous soil-borne pathogen and is a species complex with nine described varieties (Aa van der *et al.* 2000). This fungus has not been reported to cause disease on pyrethrum, hence there is no information regarding the pathogenicity or potential impact of this pathogen on commercial pyrethrum crops.

PROJECT AIMS

These studies aim to improve our understanding of this pathogen and provide baseline information to allow monitoring of changes over time.

The specific aims of this project were to assess the following aspects of *Phoma* species from pyrethrum:

1. Characterise the biological and cultural variability of *P. ligulicola* isolates and determine the variety of *P. exigua.*
2. Use phylogenetic analysis to confirm the identity of the *Phoma* species and evaluate genetic variability within the Tasmanian *Phoma* isolates.
3. Determine the baseline sensitivity of *Phoma* isolates from pyrethrum to difenoconazole, a commonly used fungicide for control of ray blight disease .
4. Characterise pathogenic variability of *P. ligulicola* isolates and determine if *P. exigua* is pathogenic to pyrethrum
5. Investigate the mating system of *P. ligulicola* isolates and determine the thallic status of *P. ligulicola* isolates from pyrethrum.

CHAPTER 2

BACKGROUND

PYRETHRUM

Tanacetum cinerariifolium is a member of the Asteraceae family which is often referred to as the Compositae family because of the composite nature of the inflorescence (Clarke and Lee 1987). *Tanacetum cinerariifolium* is a perennial herb which grows to around 75 cm high, with a spread of up to 90 cm. The grey-green leaves of mature plants are finely divided and are attached in a rosette arrangement. Solitary daisy-like flowers, 6 to 8 cm diameter, are produced on slender, elongated stems up to 90 cm long. While the flower head has the appearance of a single flower, each apparent flower is actually a compound inflorence (a capitulum) with numerous small individual flowers (florets) aggregated on a convex receptacle (Bhat 1995). In this thesis the term "florets" refers to the true flowers which collectively make up the capitulum, and the term "flower" refers to the capitulum.

Two types of florets make up the pyrethrum flower. Disc florets, with yellow corollas, cover the centre of the receptacle. Ray florets, with white corollas, form a single whorl at the outer rim of the flower (Figure 2.1). The ray florets bare a single white ligulate petal and are unisexual (no stamens), while the tubular disc florets have five petals and are bisexual. Both type of florets possess a solitary inferior ovary which produces a single seed, and together with the mature ovary wall, forms the achene (Bhat 1995).

The term pyrethrum generally refers to a number of plant species, including *T. cinerariifolium* and *T. coccinineum* (syn. *Chrysanthemum coccineum* Willd.), as well as the botanical insecticide produced from the flowers of these plants. In this thesis, the term pyrethrum will refer to the commercial insecticide produced from these plants or to the plant grown commercially in Tasmania for extraction of pyrethrins, *T. cinerariifolium*.

Figure 2.1. Pyrethrum flower with white outer ray florets and yellow disc florets in the centre.

The insecticidal components of pyrethrum are six chemical esters known collectively as pyrethrins. The six esters are divided into two groups (pyrethrins I and II) of three esters: pyrethrin, cinerin and jasmolin. The relative proportions of the six esters vary with geographic location, plant type and time of harvest and influence the effectiveness of the insecticidal properties (Crombie 1995). Pyrethrins are distributed throughout the plant tissues but are most concentrated in the florets. Approximately 94% of pyrethrins are contained in tiny oil glands which cover the outer surface of the ovary wall, and in secretory ducts within the ovary (Zito 1994). Pyrethrins are used in the production of insecticidal products such as fumigants, fly sprays, pet flea collars and mosquito coils. The pyrethrins work in combination to repel, knock down and kill a wide range of flying and crawling insects, including house flies, fleas, lice, and wasps. A synergist such as, Piperonyl Butoxide, is usually added to the insecticidal products to enhance the insecticidal action and to increase the time that the pyrethrins remain active (Kennedy and Hamilton 1995).

PYRETHRUM PRODUCTION

PRODUCTION HISTORY

Of the small number of species in the genus *Tanacetum* found to produce insecticidal substance, the two species that have been most utilised in commercial production are *T. coccineum* (Persian pyrethrum) and *T. cinerariifolium* (Dalmatian pyrethrum) (Wainaina 1995). Insecticidal use of pyrethrum is believed to have originated in Persia (present-day Iran) where the flowers of native *T. coccineum* were crushed and used to make insect powders for control of body lice (Wandahwa *et al.* 1996). The earliest commercial production of this plant species was reported to be in Armenia in 1828 (Bhat 1995). In the 1840s, the insecticidal activity of *T. cinerariifolium* was found to be more effective and it replaced *T. coccineum* as the main source of pyrethrum. Originally cultivated in its native region of Yugoslavia (Dalmatia), *T. cinerariifolium* was introduced into Eastern Africa in the 1920s and by the 1940s Kenya had become the world's largest producer of commercial pyrethrum crops. Kenya lies close to the equator and *T. cinerariifolium* is grown in cooler high altitude areas (between 1500 and 3000 m above sea level) to provide the vernalisation required for flowering . In Kenya, planting of seeds and seedlings, harvesting and drying of flowers is done by hand. Flowering occurs for approximately 10 months of the year with seasonal flushes of flower development. Individual flowers are selectively hand harvested at two to three weekly intervals according to a maturity index and dried by the growers before being sent to processing factories (Wainaina 1995). The plants are grown for a period of two to four years (Parlevliet 1974). Kenya continues to be the major producer even though *T. cinerariifolium* has been grown commercially in a number of countries including Papua New Guinea, Tanzania, Rwanda, and Australia.

PRODUCTION IN AUSTRALIA

Tasmania is the second largest producer of pyrethrum in the world with approximately 2,000 hectares of production area. Initial attempts at growing *T. cinerariifolium* on a commercial scale in Australia (Victoria, New South Wales and Tasmania) from 1930-60's were unsuccessful due to high production costs, low yields of flowers and pyrethrins, inferior planting stock and general lack of knowledge about the plants (MacDonald 1995). Improvements in planting material and production methods were gained during the 1970s and 80s through research by the Tasmanian State Government and a plant breeding program run by the University of Tasmania. The first semi-commercial crop (one trailer load) was harvested in Tasmania in 1987 (MacDonald 1995).

Tasmania lies between 41 and 44° south and has a cool temperate climate. The northwest coast, between Deloraine (41° 31' S, 146° 39' E) and Table Cape (40° 57' S, 145° 43' E), is the primary pyrethrum production area (Pethybridge *et al.* 2008c). The elevation ranges from close to sea level to around 200m above sea level, and mean annual temperatures range from 8°C in winter to 17 °C (12 year average, Commonwealth Bureau of Meteorology Data for Devonport airport, Tasmania). Pyrethrum production in Tasmania is intensively managed and seed planting, harvesting and drying of flowers is fully mechanised. Commercial varieties of pyrethrum in Tasmania have been bred for synchronous flowering (from December to January), which allows mechanical harvest. Mature flowers are cut, formed into windrows and left to dry for approximately two weeks in the fields before being processed. The dried flowers are pelletised and soaked in hexane before the solvent is removed leaving a crude oleoresin which can contain more than 35% pyrethrins. Further refining removes pigments and extraneous plant materials from the oleoresin and produces the pale refined pryrethrins which are exported to insecticidal manufacturers (Carlson 1995). Plants are around 15-18 months old before first harvest, and the life expectancy of crops is generally up to four annual harvests (MacDonald 1995).

MAJOR PATHOGENS OF COMMERCIAL PYRETHRUM CROPS

Various fungal species cause disease on Tasmanian pyrethrum crops and their prevalence varies temporally with the development stage of the plants (Pethybridge *et al.* 2005a; Pethybridge *et al.* 2003). The major fungal pathogens that affect pyrethrum production include ray blight caused by *Phoma ligulicola* Baker, Dimock & Davis v. Arx. var. *inoxydabilis* Boerema; Sclerotinia flower blight caused by *Sclerotinia sclerotiorum* (Lib.) de Barry and Botrytis flower blight caused by *Botrytis cinerea* Pers. Flower blights caused by *S. sclerotiorum* and *B. cinerea* occur during flowering in November and December. Sclerotinia flower blight causes necrosis of the ray and disc florets. The flower head remains intact and often has a bleached appearance. Botrytis flower blight has a similar appearance to Sclerotinia blight but the necrotic disc florets often fuse together (Pethybridge *et al.* 2008c). *Sclerotinia sclerotiorum* and another species, *S. minor* Jagger may also cause crown rot in pyrethrum. Crown rot appears periodically throughout the year. It causes wilting and plant death (MacDonald 1995).

Tan spot caused by *Microsphaeropsis tanaceti* R. G. Shivas, S.J. Pethybridge, & S.J. Jones produces tan coloured lesions that coalesce around the margins of leaves and contributes to defoliation in spring (Pethybridge *et al.* 2008f). The prevalence and incidence of tan spot has increased substantially since it was first described on Tasmanian pyrethrum in 2001 (Pethybridge *et al.* 2003). The extent of damage to the crop from tan spot is not yet known (Pethybridge *et al.* 2008c). Other fungal diseases found on commercial pyrethrum include winter blight caused by *Alternaria tenuissima* (Nees:Fries) Wiltshire and pink spot caused by *Stemphylium botryosum* Wallroth, but these fungi are not associated with major crop loss (Pethybridge *et al.* 2004a).

Another *Phoma* species reported to be in Tasmania (Walker and Wade 1976) and likely to be present in pyrethrum fields is *P. exigua* var. *exigua. Phoma exigua* is a ubiquitous soil-borne fungus commonly found in Eurasia and Africa (Aa van der *et al.* 1990) and also reported in Australasia and the Americas (Abeln *et al.* 2002). This fungus may be either a plant pathogen or a saprophyte

on dead plant material. It is associated with leaf and stem lesions, root rot (Boerema and Howeler 1967), damping off and dieback (Abeln *et al.* 2002) and bark necrosis (de Gruyter and Scheer 1998). *Phoma exigua* is a species complex with nine varieties currently described and two varieties (*P. exigua* var. *capsici* L.Z. Liang and *P. exigua* var. *inoxydabilis* Boerema & Vegh) which are considered to be of questionable status (Aa van der *et al.* 2000). Several varieties are acknowledged as host specific: var. *forsythiae* (Sacc.) As, Boerema & Gruyter on *Forthsythia* hybrids, var. *linicola* (Naoum. & Vass.) Maas associated with flax (*Linum usitatissimum*), var. *heteromorpha* (Schulzer & Sacc.) Nordel. & Boerema on oleander (*Nerium oleander*), var. *noackiana* (Allesch.) Aa, Boerema & Gruyter on beans (*Phaseolus vulgaris*) (Abeln *et al.* 2002). The remaining *P. exigua* varieties are known to be plurivorous and capable of infecting multiple host species. The most notable plurivorous variety, *P. exigua* var. *exigua*, the type species of *Phoma* sect. *Phyllostictoides* Sacc. ex Sacc., has been isolated from more than 200 host genera including some hosts that are infected by the more host-specific *P. exigua* varieties (Aa van der *et al.* 2000). This variety may produce necroses on stems and leaves, and rot at bases of stems and leaves and on fleshy roots and tubers. Examples include gangrene of potatoes (*Solanum tuberosum*), foot rot of lettuce (*Lactuca sativa*), root rot of carrots (*Daucus carota*) and speckle disease of beans (*Phaseolus vulgaris*) (Boerema *et al.* 2004). The other *P. exigua* varieties and their primary hosts are: var. *diversispora* on cowpea (*Vigna unguiculate*) and beans (*P. vulgaris*), var. *populi* on cultivars of poplars (*Populus nigra* and *P.* × *euramericana*), *var. lilacis* on lilac (*Syringa vulgaris*) and var. *viburni* on viburnum (cultivated *Viburnum* spp.). No *P. exigua* varieties have been associated with a teleomorph stage (Boerema *et al.* 2004).

In Tasmania, *P. exigua* var. *exigua* has been associated with gangrene on stored potato tubers (Walker and Wade 1976). Potatoes are sometimes grown in rotation with pyrethrum hence it is possible that this tuber-and soil-borne fungus is present either in the soil or on volunteer (self-sown) potatoes after potato harvest. So while it is likely that *P. exigua* var. *exigua* occurs in pyrethrum fields this fungus has not been reported to infect *Tanacetum* or *Chrysanthemum* species.

RAY BLIGHT DISEASE

The most serious fungal disease currently limiting Tasmanian pyrethrum production is a foliar dieback and flower blight caused by *P. ligulicola* var. *inoxydabilis*. This disease is commonly known as ray blight disease. This fungus is soilborne and ray blight disease appears to have become more prevalent after the change in industry practice from planting of clonal splits to direct seeding in the late 1990s. Ray blight disease is manifest as (i) a severe dieback of emerging stems and leaves in spring and (ii) a disease of flowers.

Figure 2.2. Necrosis of the growing tips progressing down the stem and defoliation are symptoms of the spring phase ray blight caused by *Phoma ligulicola* var. *inoxydabilis*.

Figure 2.3. Characteristic ray blight symptoms on pyrethrum flowers: drooping necrotic flower buds and pinching between the necrotic lesion and healthy area below the peduncle.

In spring, foliar symptoms include dieback of leaves and growing points towards the stem. Leaf lesions typically start at the leaf margin which can expand to cover entire leaves. Lesions from leaves often extend onto stems and cause stem girdling. Severe foliar infection can cause extensive defoliation, stunted plants and plant death (Figure 2.2). Leaf and stem lesions are most prominent during early spring but also occur during autumn and winter (Pethybridge *et al.* 2003). Infection of the flowers is manifest as necrotic lesions often extending 20 to 30 mm down the stem from the peduncle of infected unopened buds and flowers, causing a distinctive droop or 'shepherd's crook' appearance. Another characteristic of this disease is the clear delineation and constriction at the junction of nectrotic tissue (below the peduncle) and healthy stem tissue (Figure 2.3). The incidence of these symptoms is highest in the main flowering period (November to December) but they can be found at any time that flowers are present (Pethybridge *et al.* 2008c). Pycnidia are readily found within necrotic tissue on diseased plants, but perithecia have not been observed in pyrethrum fields (Pethybridge and Hay 2001). Conida and mycelia, in and on plant residues, are both sources of inocula in pyrethrum fields (Figure 2.4). Infection is initiated by penetration of hyphae or germinated conidia through the epidermis of plant tissue (Pethybridge and Wilson 1998). Since *P. ligulicola* was first reported in Tasmania in 1995 (Pethybridge and Wilson 1998) the

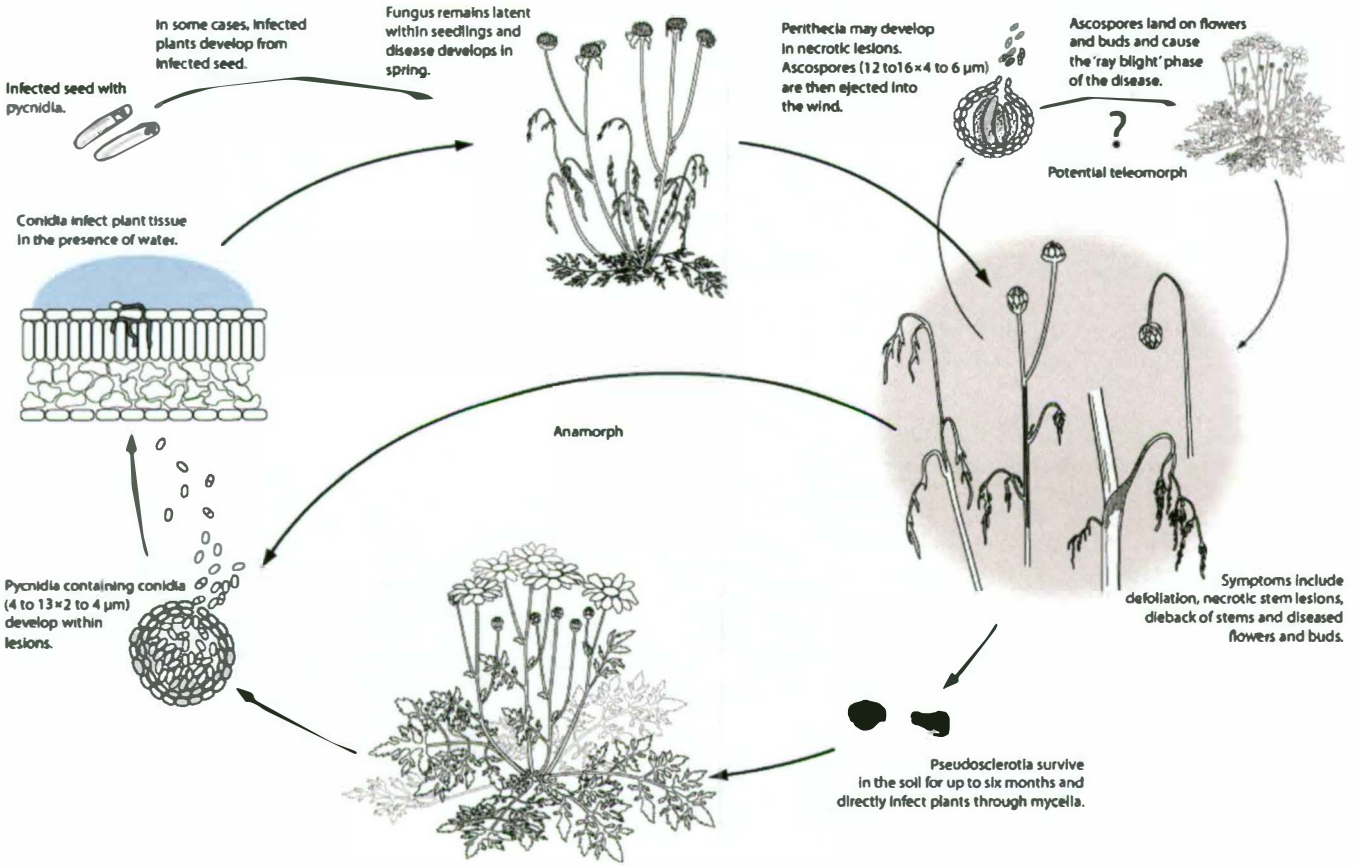

Figure 2.4. Life cycle of *Phoma ligulicola* var. *inoxydabilis*, the cause of ray blight disease in Tasmanian pyrethrum fields. The contribution of a teleomorph stage is at present unknown. (Illustrated by Vickie Brewster) (Pethybridge *et al.* 2008c)

pathogen has become prevalent in commercial pyrethrum crops. The disease is believed to have been inadvertently introduced into Tasmania on infected seed (Pethybridge *et al.* 2008c). This fungal species has also been associated with foliar disease in commercial pyrethrum crops in Kenya, Tanzania and Papua New Guinea (Pethybridge *et al.* 2004a). In the absence of appropriate disease control, this foliar disease has resulted in early termination of crops and total loss of yield (Pethybridge *et al.* 2005b).

RAY BLIGHT ON CHRYSANTHEMUM

Phoma ligulicola also causes ray blight of chrysanthemum. Ray blight of chrysanthemum flowers was first reported in 1904 in North Carolina (Stevens 1907). The fungus remained relatively confined to the south-eastern states of USA until the late 1950s. With the expansion of the chrysanthemum industry, *P. ligulicola* spread to other areas when infected cuttings and plants with latent infection were transported to northern states of the USA (Baker *et al.* 1949). The disease spread to other parts of the world including England and Japan when infected chrysanthemum plants were exported from the USA (Baker *et al.* 1961). *Phoma ligulicola* was first reported on chrysanthemum in Australia in the Sydney metropolitan area in New South Wales (Anonymous 1956).

On chrysanthemum, necrosis often starts on one side of infected flowers and spreads until the entire flower is necrotic. Infected florets are often stuck together. The necrotic stem below the peduncle and drooping flower head observed in pyrethrum are also characteristic of the disease on chrysanthemum. While the disease is visually prominent on the flowers, lesions on foliage contribute to damage and to spread of the disease. Leaf lesions are irregular black blotches that may be 2 to 3 cm wide. Pycnidia, and less frequently perithecia, may be present on symptomatic tissue (Baker *et al.* 1961).

On chrysanthemum, infection by *P. ligulicola* is favoured by senescing and wounded plant tissue (Baker *et al.* 1961). Petals are easily penetrated by hyphae leading to symptoms of pink spots followed by petal decay as the

disease progresses. In field crops of chrysanthemum, disease is confined to localized foci during warmer months with splash dispersed conidia. Ascospores are produced in the cooler months and dispersed when flowers are developing. Ascopores can be forcefully discharged and become airborne, spreading new foci onto higher plant parts and developing flowers (Baker *et al.* 1949).

CLASSIFICATION AND IDENTIFICATION OF *PHOMA* SPECIES

The *Phoma* genus belongs to the Coelomycete genera and contains over 2000 described species (Sutton 1980) occurring mainly on plants and soil (Leath 1992). To facilitate identification at the species level, the genus is subdivided into nine sections primarily based on pycnidia and conidia morphology, but also on teleomorph associations. *Phoma ligulicola* is in the *Phyllostictoides* section of the *Phoma* genus. This section is delimited from the other eight *Phoma* sections by the occasional production of two-celled (septate) conidia. *Phoma ligulicola* is the anamorph of *Didymella ligulicola* (Baker, Dimock and Davis) v. Arx, (Boerema 1997). This fungus was first described on chrysanthemum as *Ascochyta chrysanthemi* (Stevens 1907). Based on the teleomorph stage the fungus was later named *Mycosphaerella ligulicola* (Baker *et al.* 1949). The name of this species was revised as *Didymella chrysanthemi* (anamorph *P. chrysantemi*) in the CMI description (Punithalingam 1980) and again in 1990 as *Didymella ligulicola* (anamorph *P. ligulicola* Boerema) along with the identification of two varieties (Aa van der *et al.* 1990).

Approximately 200 *Phoma* species have been studied in detail and revised for their taxonomic status. Individual *Phoma* species are generally either a) plurivorous and weakly parasitic or opportunistic on wounds or saprophytic on dead plant material; or b) specific pathogens of cultivated plants (Aa van der *et al.* 1990). Economically important plant pathogenic *Phoma* species include *P. medicaginis* var. *macrospora* (black stem of lucerne), *P. destructiva* var. *destructiva* (fruit rot, leaf and stem blight of tomato and paprika), *P. andigena* (black potato blight), *P. lingam* (dry rot of cultivated *Brassica* spp.) and *P. betae* (black leg of beet and spinach) (Boerema *et al.* 2004) .

The *Phoma* genus is considered to be taxonomically problematic because of the difficulties in clearly distinguishing one species from another. Additionally, because a large number of *Phoma* species are known only from their asexual (anamorphic) states, the current taxonomic system is lacking in phylogenetic clarity (Torres *et al.* 2005). Identification and classification of *Phoma* species has traditionally been based on morphology (*in vitro* cultures and *in vivo* samples) and host substrate. In the past, some fungal species were described on the basis of a restricted number of characters, and in some cases solely on host or substrate. For example, *P. herbarum*, the type species of the *Phoma* genus, was once thought to occur only on stems of herbaceous plants but was later found on a diverse range of substrates including soil, water and air (Boerema *et al.* 2004). As a result, this fungus was mistakenly described under numerous names according to the substrate it was isolated from. So while host, substratum and pathogenic traits are useful for differentiation of taxa, application of these criteria is likely to be more reliable when used in conjunction with a range of *in vitro* cultural and morphological characteristics obtained under controlled conditions (Aa van der *et al.* 1990). Features used to classify fungal species *in vitro* include mycelial growth rates on various agar media, colony colour, aerial mycelium colour and texture, and sporulation. Gross morphology and biochemical processes are influenced by environmental conditions including temperature, light and substrate (Leath 1992). Subsequently, a variety of media and incubation conditions are often used to provide a comprehensive account of cultural characteristics.

Some species of *Phoma* produce a colourless diffusible metabolite. The metabolite is known as metabolite 'E' because it was first detected in *Phoma exigua* var. *exigua* (Boerema and Howeler 1967). The presence of metabolite E is tested by application of a drop of sodium hydroxide (NaOH) to margins of cultures growing on various agar media. The alkaline NaOH causes the colourless metabolite to oxidise and form pigment α (blue-green), which later changes to pigment β (red to reddish-brown). Initially used as a diagnostic tool for differentiation of varieties of *P. exigua* (Boerema and Howeler 1967), this test is also used to differentiate the two varieties of *P. ligulicola*, vars. *ligulicola*

and *inoxydabilis* (Aa van der *et al.* 1990). The metabolite has antibiotic properties and is believed to give fungi a competitive advantage over bacteria and other fungal species in colonisation of substrates (Logan and O'Neil 1970). Additional features used to differentiate the two *P. ligulicola* varieties are mycelial growth rates on oatmeal agar (OA) and malt extract agar (MA) and the type of reproductive structures they produce in culture (de Gruyter *et al.* 2002). Both the sexual (teleomoph) and asexual (anamorph) reproductive stages have been identified on diseased plant material (*in vivo*) for both varieties of *P. ligulicola*. However *P. ligulicola* var. *ligulicola* is reported to produce only the anamorph in agar cultures, while var. *inoxydabilis* produced both stages in agar cultures (Aa van der *et al.* 1990).

Molecular techniques are frequently used to supplement traditional methods of fungal recognition and taxonomy (Bruns *et al.* 1991; Lindqvist-Kreuze *et al.* 2003; Reddy *et al.* 1998). Additionally, information of specific genomic regions can be used for detection and diagnosis of the causal agents of plant diseases (Lees *et al.* 2005), phylogenetic analysis and inference of ancestral relationships (Cooke *et al.* 2000; Peever *et al.* 2007), characterisation of population variability (Balmas *et al.* 2005), and detection of particular traits such as fungicide resistance (Avenot *et al.* 2008) or modes of reproduction (Kauserud and Schumacher 2001). While detection and identification are fundamental to the development of methods for disease management, knowledge of the genetic composition of pathogen population can provide important insights into the potential for a pathogen to develop new traits such as fungicide resistance or virulent pathotypes (Bruns *et al.* 1991). Development of durable disease management strategies therefore requires knowledge of the genetic structure of a pathogen population.

Published information on the genetic composition of *P. ligulicola* is limited. The internal transcribed spacer (ITS) regions (ITS1 and ITS2) and the 5.8S ribosomal RNA gene have been sequenced for 15 *P. ligulicola* isolates obtained from pyrethrum and chrysanthemum (Pethybridge *et al.* 2004b). Primers specific to *P. ligulicola* have been designed (Pethybridge *et al.* 2004b) and used

for diagnostic purposes such as detection of the pathogen in pyrethrum seed (Pethybridge *et al.* 2006a). Random amplified polymorphic DNA (RAPD) analysis has also been used to examine genetic variability within a population of *P. ligulicola* (Pethybridge *et al.* 2005c). Random amplified polymorphic DNA (RAPDs) are a PCR-based technique that generates multilocus data from random DNA fragments throughout the entire genome (Williams *et al.* 1990). RAPD-PCR has the potential to survey entire genomes for random variability and may detect genetic differences that are not evident in single-gene comparisons (Bakonyi and Justesen 2007; Hseu *et al.* 1996). For example, RAPD fingerprinting has been used to separate isolates of *Ganoderma lucidum* with identical or nearly identical ITS sequences (Hseu *et al.* 1996). Previous studies found higher levels of intraspecific variability among *P. ligulicola* isolates for the RAPD analysis (Pethybridge *et al.* 2005) and AFLP (Jason Scott *pers. comm.*) than for sequences of the ITS region (Pethybridge *et al.* 2005c; Pethybridge *et al.* 2004b).

RAY BLIGHT DISEASE MANAGEMENT

DISEASE MANAGEMENT ON CHRYSANTHEMUM

In commercial chrysanthemum flower production, ray blight disease affects plants grown in the field and in greenhouses (Engelhard 1984). The flowering stage is particularly susceptible to infection. Because the chrysanthemum industry produce flowers continually throughout the year, the pathogen is provided with a susceptible host for most of the year (Baker *et al.* 1961). Commercially produced cuttings are the main method of propagation in this industry. Cuttings in propagation beds are susceptible to infection from soil borne inoculum through cut surfaces and infected cuttings may fail to develop roots. The fungus may colonise the surfaces of roots on cuttings but the cuttings may appear symptomless. As such they are a major source of disease distribution when diseased plants with no apparent symptoms are transported and sold through wholesale nurseries (Chesters and Blakeman 1966). Methods

used to control ray blight disease include propagation with disease free plant materials and wide spacing of plants to improve ventilation (Baker *et al.* 1949). Soil treatment, preventative fungicide application, removal and destruction of crop debris and the use of watering methods that do not wet foliage and flowers are also strategies for disease management (Baker *et al.* 1961; Strider 1994). Historically, fungicides reported to be effective for ray blight on chrysanthemum include zinc ethylene-bis-dithiocarbarnate, phenyl mercury triethanol ammonium lactate and dichloronaphthoquinonc, although the latter two caused severe petal damage at high concentrations (Baker *et al.* 1949). More recent reports of effective fungicides include iprodione, propiconazole, vinclozolin (Engelhard 1984), benomyl, chlorothalonil and mancozeb (Strider 1994). The use of less susceptible varieties of chrysanthemum has also been reported (Baker *et al.* 1949; 1961; Engelhard 1984) but there is no published information on controlled testing of varietal susceptibility to *P. ligulicola* var. *ligulicola*.

DISEASE MANAGEMENT ON PYRETHRUM

No sources of resistant plant material have been identified for the Tasmanian pyrethrum industry and fungicides currently provide the basis of ray blight disease control. In Tasmania, pyrethrum is propagated from seed which is sown directly into fields. Reduction of initial inoculum in planting material is achieved by fungicide treatment of seed crops during the growing season, testing of seed for incidence of *P. ligulicola* and treatment of seed with fungicides prior to planting (Pethybridge *et al.* 2006a). The cultural methods used for disease control by the chrysanthemum industry are not viable or practical for the Tasmanian pyrethrum industry due to differences in production systems. For example, pyrethrum is grown only in fields, not in greenhouses; and the crop is watered either by natural rainfall or with irrigation systems which cause the foliage and flowers to become wet. Additionally, in the Tasmanian pyrethrum production system, flowering is more synchronised and most flowers are produced from November to January. Hence the host plant is in the most

susceptible stage for infection by *P. ligulicola* for a shorter period of time in this system. This means that relatively large geographic areas of the host are at the susceptible stage simultaneously. With the exception of adjusting plant density to improve ventilation, there are no practical means of controlling the environmental conditions at any stage of the crop. Consequently, there is potential for large outbreaks of the disease to occur under the right conditions.

Although ray blight disease can affect flowers of Tasmanian pyrethrum, this phase is considered to be less important than dieback of leaves and developing flower stems in early spring. The reduction in foliage and newly forming flowering stems in spring correlates with reduced flower and pyrethrin yields (Pethybridge *et al.* 2005b). A fungicide program was implemented by the Tasmanian pyrethrum industry in 2001 after ray blight epidemics caused severe crop losses. To minimise development of fungicide resistance the pyrethrum industry (i) alternate fungicides from different chemical groups, (ii) uses mixtures of fungicides with different modes of action and (iii) limits the number of fungicide applications in a season. The fungicide program includes a single application of azoxystrobin (as Amistar®; Syngenta Australia), followed by two applications of a mixture of chlorothalonil (as Bravo 720®; Syngenta Australia) and difenoconazole (as Score®; Syngenta Australia) during spring (Pethybridge *et al.* 2005b). Initial application coincides with emergence of the flowering stems in spring (Pethybridge *et al.* 2003). Additionally, tebuconazole (as Folicur®) is applied twice in the period from November until harvest in December/January to control White flower mould caused by *Sclerotinia sclerotiorum* and Botrytis flower blight caused by *Botrytis cinerea* (Pethybridge *et al.* 2008e).

Tebuconazole and difenoconazole are sterol demethylation inhibitors (DMIs). These systemic fungicides act by inhibiting the biosynthesis of ergosterol, a key ingredient in fungal membranes (Köller 1988). DMIs are highly site specific however and this single-site mode of action makes them more prone to resistance development than fungicides that are multi-site inhibitors (Scheinpflug 1988a). The mode of action within fungicide chemical groups is

generally very similar. Hence resistance to one member of a chemical group often signifies resistance to other fungicides in that group. This is known as cross-resistance and it has been observed for several DMIs and in various pathosystems (Golembiewski *et al.* 1995; Köller *et al.* 1991). While resistance to DMIs is thought to be through polygenic or multistep acquisition, little is known about the molecular or biochemical mechanisms responsible for resistance development (Delye *et al.* 1997). Possible explanations for DMI resistance include efflux of the toxicant from fungal cells, over production of the P-450 14α-demethylase enzyme or alteration of fungal membrane structure (Henry 1991).

Azoxystrobin is a member of the strobilurin or quinone outside inhibitors (QoI) fungicide group. Strobilurin fungicides have a single-site mode of action. These fungicides inhibit mitochondrial respiration by binding to the Qo site (the outer, quinone oxidixing pocket) of the cytochrome bc1 enzyme complex (Gisi *et al.* 2002). Reduced sensitivity to strobilurins has developed in a number of plant-pathogenic fungi and resistance can develop rapidly (Bartlett *et al.* 2002). For example, field efficacy was lost for the control of powdery and downy mildews in cucumbers only two years after the first introduction of members of this fungicide group, despite following manufacturer recommendations (Ishii *et al.* 2001). A G143A mutation on the cytochrome b gene has been associated with practical resistance to azoxystrobin in *Alternaria* spp. (Avenot and Michailides 2007).

Chlorothalonil is a broad spectrum contact or protectant fungicide. It is a multisite inhibitor and the mode of action involves chlorothalonil reacting with sulfhydryl groups present in proteins, such as molecules of glutathione (Roberts and Hutson 1999). Multisite inhibitors require multiple genomic modifications to develop resistance (Köller and Scheinpflug 1987). Hence resistance development is slower than with chemicals with a single site mode of action. Alternation of systemic fungicides with protectant broad spectrum fungicides such as chlorothalonil is recommended for resistance management of dicarboximides (Löcher 1988) and DMIs (Urech 1988).

PATHOGEN AND HOST INTERACTIONS

Breeding disease resistant plant material is a potential disease control strategy that could complement other forms of disease management for the pyrethrum industry and reduce the reliance on fungicide use. Knowledge of the susceptibility or resistance of the host, and the pathogenicity (aggressiveness and virulence) of the pathogen is crucial for the success of resistance breeding programs. The interactions between hosts and pathogens indicate that they evolve together (Flor 1971). For each gene that regulates a resistance response in the host there is a corresponding gene in the pathogen that governs pathogenicity. Genetic mutations in one are normally balanced by a corresponding genetic adjustment in the other. This results in a constantly changing pathosystem equilibrium as each genetic adaptation in host resistance is matched by a change in pathogenicity (Flor 1971). The degree of pathogenicity to a particular host, and the extent of host susceptibility or resistance to a pathogen, are largely due to inherited characteristics and can vary from one individual to another. A single fungal isolate may be able to infect all tested cultivars, while another isolate of the same species may be able to infect just one cultivar or a certain group of cultivars. Similarly, host resistance can be effective against all tested individuals of a fungal population (horizontal resistance) or against specific individuals (vertical resistance) and the two types of resistance are not mutually exclusive (Van der Plank 1968). Vertical resistance mechanisms are generally monogenic (encoded at single loci), sometimes at a few loci (oligogenic), and these resistance mechanisms operate against a specific genetic component of the pathogen. Horizontal resistance mechanisms are usually inherited polygenically, with traits encoded by many loci (Deadman 2006). Vertical resistance components are generally easier to identify, manipulate and incorporate into plant breeding than horizontal genetic traits, but horizontal resistance is considered to be more stable (Flor 1971).

Assessment of pathogen-host interactions can be done by quantifying the amount of disease that develops over a given period of time, under a certain set of conditions. Such assessments may include the number of lesions on a specified area (Khan *et al.* 1999) or the proportion of the host with disease symptoms (Kaiser *et al.* 1997), the area of necrotic tissue (Chen *et al.* 2005) or the time it takes for infection and disease symptoms to occur (Evans *et al.* 1997). Pathogen virulence is defined as the amount of disease that a pathogen can cause and a virulent pathogen is capable of causing a relatively severe amount of disease (Agrios 1997). Use of several isolates from the agronomic region, whose pathogenicity has been characterised, is recommended for effective resistance screening because testing of just one or few isolates may fail to detect cultivar-isolate interactions associated with vertical resistance (Allingham and Jackson 1981; Scharen and Krupinsky 1970).

CHARACTERISATION OF PATHOGENICITY

Characterisation of the pathogenic variability among isolates collected from the production area of interest is an important component of plant breeding for resistance. It allows identification of virulent isolates or pathotypes that can then be used to test cultivars for susceptibility and resistance, and to assess the risk of susceptibility developing in existing sources of disease resistance (Arabi and Jawhar 2007). A pathotype is defined as a group of isolates that are identical in their virulence or avirulence to a given set of host cultivars or varieties (Brown 2006). Pathogenicity and host susceptibility are characterised and measured in various ways including field trials conducted over multiple years (Kong *et al.* 1997), greenhouse studies (Chen *et al.* 2005; Kaiser *et al.* 1997) and growth chamber assays of detached plant parts under highly controlled conditions (Benedikz *et al.* 1981; Blakeman and Dickinson 1967).

HOST RANGE OF PHOMA LIGULICOLA

The two varieties of *P. ligulicola* are reported to vary in their host range. *Phoma ligulicola* var. *ligulicola* isolates from florists' chrysanthemum (*Dendrathema* × *grandiflorum* Kitam., formerly known as *Chrysanthemum* × *morifolium* Ramat.) were reported to infect annual chrysanthemum (*Chrysanthemum carinatum* Schousb.), endive (*Cichorium endivaia* L.), globe artichoke (*Cynara scolymus* L.), rudbeckia (*Rudbeckia hirta* L.), zinnia (*Zinnia elegans* L.), sunflower (*Helianthus annuus* L.), dahlia (*Dahlia variabilis* Desf.) and lettuce (*Lactuca sativa* L.) (Chesters and Blakeman 1967). In more recent accounts, *P. ligulicola* var. *ligulicola* is referred to only as 'a specific pathogen of florists' chrysanthemum' (Aa van der *et al.* 1990; de Gruyter *et al.* 2002). No reports were found regarding the specific pathogenicity of *P. ligulicola* var. *ligulicola* to *T. cinerariifolium* however it has been reported to colonise roots of an unspecified pyrethrum species (Chesters and Blakeman 1966).

Phoma ligulicola var. *inoxydabilis* has been isolated from wild and cultivated Compositae including feverfew (*Tanacetum parthenium* L.), and zinnia (Aa van der *et al.* 1990). A study of the host range of *P. ligulicola* var. *inoxydabilis* isolated from commercial Tasmanian *T. cinerariifolium* (Pethybridge *et al.* 2008a) found that, with the exceptions of *D.* × *grandiflorum* and *C. carinatum*, this *P. ligulicola* variety was not able to infect those species reported by Chesters and Blakeman (1967), including zinnia which had been reported more recently (Aa van der *et al.* 1990; Boerema *et al.* 2004). Variation in the genotype and phenotype of plant material and isolates tested by Pethybridge (2008a) and those examined by (Aa van der 1990) may account for the discrepancy in pathogenicity of *P. ligulicola* var. *inoxydabilis* to zinnia. Additionally, there are no details of the method used by Aa van der (1990) to confirm pathogenicity.

REPRODUCTIVE MECHANISMS OF *PHOMA LIGULICOLA*

For many fungal species the reproductive phase has a major influence on the pathogens ability to disperse, cause infection and survive. *Phoma ligulicola* belongs to the Ascomycete group of fungi whose members are characterised by the formation of asci (Ulloa and Hanlin 2000). *Phoma ligulicola* may have two distinct reproductive phases, a sexual cycle (teleomorph) and an asexual (anamorph) cycle. The teleomorph and anamorph stages have been described for the two *P. ligulicola* varieties (Aa van der *et al.* 1990; de Gruyter *et al.* 2002). However the teleomorph (*Didymella ligulicola)* has not been detected in *P. ligulicola* var. *inoxydabilis* cultures studied *in vitro* or found on diseased plant material collected from pyrethrum crops (Pethybridge and Hay 2001; Pethybridge *et al.* 2003; Pethybridge and Wilson 1998).

Different spores are produced during the teleomorph and anamorph stages. During the sexual lifecycle stage, ascospores are produced in perithecium while in the asexual stage conidia are produced in pycnidia. Both types of spores are the principal means of pathogen dispersal and disease spread. The two types of spores are dispersed in different ways and this has important implications for disease spread. Ascospores can be discharged forcefully into the air where they can become airborne and transported relatively long distances by wind. They are primary sources of inoculum for new infections in other areas of the crop or in nearby crops. Conidia are carried mainly by water runoff or splash dispersal and travel relatively short distances. They cause localised infection and increase the amount of disease (disease intensity) (Baker *et al.* 1949; McCoy *et al.* 1972).

Sexual reproduction involves the fusion of two genetically different nuclei and results in novel genotypes. Every cycle of sexual reproduction can potentially generate a number of new genotypes through recombination (Leung *et al.* 1993). While favourable traits may be enhanced, diluted or maintained through sexual reproduction (Van der Plank 1982), new genetic combinations can enhance the overall adaptation of the species to changing environments (Moore and Frazer 2002) and may increase the pathogen's ability to overcome host resistance and to develop fungicide resistance (Barve *et al.* 2003). Asexual

reproduction does not involve genetic recombination. With the exception of mutations, asexual reproduction results in offspring that are genetically identical to the parent fungus and each other (Milgroom 1996). Adaptation of asexual fungal populations occurs through acquisition of successive favourable mutations (Leung *et al.* 1993). Selection pressure also influences the adaptation of asexual populations and changes the frequency certain genotypes and phenotypes (Van der Plank 1982).

REPRODUCTIVE SYSTEMS OF FUNGI

Fungi that produce sexual spores may be either homothallic (self-fertile) or heterothallic (self-sterile). Homothallic fungi can reproduce sexually on their own. Heterothallic fungi require two individuals of different mating types to produce sexual spores (Alexopoulos *et al.* 1996). In heterothallic fungi, mating types are specified by two highly divergent alleles at a single mating type (*MAT)* locus. Alleles are called idiomorphs in mating type systems because, although they occupy the same locus on their chromosome, they are highly variable and exhibit very low sequence homology (Metzenberg and Glass 1990). All known heterothallic ascomycetes have only two mating types (Coppin *et al.* 1997). The mating type of an isolate depends on which of the two idiomorphs it has at the *MAT* gene locus. Idiomorphs containing the alpha box region are designated as *MAT-1* and idiomorphs with high mobility group (HMG) motifs are *MAT-2* (Arie *et al.* 1997; Turgeon and Yoder 2000). In heterothallic species mating type 1 (*MAT-1*) isolates contain only the alpha box DNA binding motif and mating type 2 (*MAT-2*) isolates contain only the high mobility group (HMG) motif. In heterothallic fungi, successful mating only takes place between cells or mycelia that have different idiomorphs (either *MAT-1* or *MAT-2*) at the mating type locus (Metzenberg and Glass 1990). Fungi with primary homothallism exhibit no mating type and contain both the alpha and HMG motifs within the mating gene and are designated *MAT-1/2* (Turgeon and Yoder 2000).

Pseudohomothallic fungi incorporate two nuclei of opposite mating types into some or all spores and produce a self-fertile thallus capable of completing the sexual cycle. This is also called secondary homothallism because 'normal' heterothallic behaviour is effectively circumvented and as a result the fungus behaves like it is homothallic (Alexopoulos *et al.* 1996). *Neurospora tetrasperma* often produce asci with four ascospores, each containing two nuclei of opposite mating type. On germination, these ascopores produce a heterokaryotic culture that is functionally homothallic (Metzenberg and Glass 1990). *Botryotinia squamosa* is an example of a mating system with secondary homothallism. The self-fertility found in otherwise heterothallic *B. squamosa* isolates is attributed to the presence of genetically different nuclei in vegetative cells such as mycelia, conidia and sclerotia (Lorbeer 1985). Pseudohomothallic behaviour in filamentous ascomycetes has also been attributed to the presence of two nuclei of each mating type in a single conidium (Nelson 1996).

VEGETATIVE COMPATIBILITY AND MATING TYPE GENES

Sexual reproduction involves transfer of nuclei from one fungal isolate (or hyphae) to another, followed by fusion of the paired nuclei (karyogamy) and meiosis. The transfer of nuclei from one hyphae to another involves fusion of two hyphae (anastomosis). Hyphal fusion is generally regulated by vegetative compatibility genes, but may also be influenced by mating type genes (Loubradou and Turcq 2000).

Vegetatively compatible isolates grow into each other without altering their morphology and form a stable heterokaryon (Leslie 1993). If the colonies involved are not vegetatively compatible the cells immediately involved typically die. This prevents transfer of nuclei and other organelles between incompatible strains. Anastomosis is not limited to sexual reproduction and is an essential function of ascomycete mycelia. Hyphal fusions commonly occur in individual mycelium and enable nutrients and molecules to be transported throughout the colony (Moore and Frazer 2002). Vegetative compatibility and

sexual reproduction are interdependent functions in sexual reproduction, but they are essentially two separate systems. Vegetative compatibility does not necessarily guarantee mating compatibility and subsequent sexual reproduction. Similarly, sexual reproduction does not always require vegetative compatibility. Colonies that are vegetatively incompatible, and form a barrage, may still be sexually (mating type) compatible because cell mortality does not extend to fused trichogynes even though the fused vegetative cells die. Trichogynes are specialised hyphae that fuse with hyphae or conidia of the opposite mating type to form perithecia (Metzenberg and Glass 1990). Perithecia can form on one or both sides of the barrage zone. For example pairing of *Podospora anserine* isolates resulted in perithecia on both sides of the barrage while for *Gelasinospora tetrasperma* isolates, perithecia formed on only one side of the barrage due to migration of nuclei in one direction only (Moore and Frazer 2002).

CHAPTER 3

BIOLOGICAL AND CULTURAL VARIABILITY OF *PHOMA* SPECIES FROM PYRETHRUM AND CHRYSANTHEMUM

INTRODUCTION

Cultural variability is inherent in some *Phoma* species, with individual isolates of a single species showing notable variability in cultural appearance and morphology (Aa van der et al. 2000). Phenotypic differences can occur due to geographic separation and host-interactions with the pathogen, and may be expressed as cultural and biological variability within species and varieties. Alternatively, differences in morphological characteristics between *Phoma* species can be very slight, making identification difficult (Sutton 1980). Criteria used to differentiate *Phoma* species frequently overlap making clear distinction between species problematic. To facilitate correct identification of fungal isolates collected from diseased plants in the field it is first necessary to gain an understanding of the nature and extent of cultural and morphological variability. Additionally, it is important to understand within-species variability of pathogen populations and to identify characteristics which may enhance their ability to survive, reproduce and cause disease.

Reproductive structures are the key morphological features used to identify and classify many fungal species. Sexual reproductive structures are of limited use for identification of *Phoma* species because relatively few have documented teleomorphs (Boerema *et al.* 2004). Hence anamorph reproductive structures, pycnidia and conidia, are important morphological features for *Phoma* species identification. Both pycnidia and conidia can vary considerably in shape and size between species and within species or populations. Production of reproductive structures is influenced by environmental factors. For example, conidium size and per cent septation of *P. ligulicola* is in inversely proportional to temperature (McCoy *et al.* 1972). To minimise variability due to extraneous factors, cultures need to be studied under well controlled conditions.

This chapter examines morphological and cultural variability of Tasmanian *Phoma* isolates obtained from pyrethrum. Two scales of *P. ligulicola* isolate population were assessed, a within-field population and a regional between-field population. The *P. exigua* isolates were also included in this study for identification and comparison to *P. ligulicola* isolates. An additional five *P. ligulicola* var. *ligulicola* isolates sourced from chrysanthemum were also assessed to allow comparison of Tasmanian isolates with reference isolates.

The aims of this study were to:

1. Characterise the *P. ligulicola* isolates obtained from pyrethrum in terms of cultural characteristics, mycelial growth rates and conidial dimensions.
2. Confirm the species and varieties of *Phoma* species obtained from pyrethrum.
3. Compare the Tasmanian *P. ligulicola* var. *inoxydabilis* isolates to *P. ligulicola* var. *ligulicola* isolates sourced from chrysanthemum in terms of cultural and morphological characteristics.

METHODS AND MATERIALS

COLLECTION OF ISOLATES

Two field populations of *Phoma* species were studied by sampling from pyrethrum crops with ray blight symptoms. The between-field population was sourced from the culture collection at the Tasmanian Institute of Agricultural Research, Cradle Coast Campus, Burnie, Tasmania. These 58 isolates were collected from 52 pyrethrum crops on the north coast of Tasmania, from Wynyard (40° 59' S, 145° 43' E) to Scottsdale (41° 09' S, 147° 30' E), in 2003 and 2004. Isolations were made from diseased stems, leaves and buds. The within-field population was collected from a single field at Wesley Vale (41° 11'S 146° 27' E) on the central north coast of Tasmania in October 2006.

These isolates were collected from nontreated plots in a fungicide trial. Fifty samples of plant material with ray blight symptoms (necrotic stems and buds) were collected from each of three plots. The total area of the trial was 65 m long and 19 m wide, and the plots were 5 m long and 4 m wide. Each plot was surrounded with a 1 m buffer zone. Plot 1 was located 6 m in from the western boundary on southern boundary of the trial site. Plot 2 was located 8 m east north-east from plot 1 and plot 3 was located 26 m to the north of plot. These same plots were sampled again during December 2006 and fifty samples of plant material with symptoms of ray blight were collected from each of the three plots. Small pieces of plant material (< 2 mm^3) were excised from the junctions of healthy and necrotic tissue, placed on 2% water agar (WA) and incubated at 20°C in darkness. After 48-72 h, single hyphal tips were isolated from the resulting fungal growths then transferred to potato dextrose agar (PDA, 39 g in 1L distilled water; Amyl laboratories, Melbourne, Australia) and incubated at 20ºC in darkness.

Representative *P. ligulicola* isolates sourced from New South Wales (DAR 70020), USA (ATCC 10748), Germany (DSMZ 63133 and 62547) and New Zealand (ICMP 2287 and 10673) were included in this study for verification and comparison. Isolate DAR 70020 was originally collected from a pyrethrum crop at Forth (41° 11 ' S, 146° 15 ' E) in Tasmania. Hence this isolate was included in between-field population for these studies. The five isolates from Germany, USA and New Zealand are referred to as the 'foreign isolates' population throughout this thesis.

Four isolates tentatively identified as *P. exigua* were also isolated from pyrethrum showing typical symptoms of ray blight disease while the within-field and between-field populations were being collected (two from each population). These four cultures were considered as a separate *P. exigua* population in this study.

All isolates were stored by excising small blocks (5 to 10 mm^2) of mycelia grown on PDA from each isolate and then maintained in sterile distilled water at 4°C. Isolates were also maintained at -80°C on dried 5 mm PDA plugs. The PDA plugs were removed from 7 to 14 day old cultures grown on PDA, dried in a laminar flow cabinet for 16 to 20 h and then placed into 1.2 ml Iwaki plastic cryogenic vials (Asahi Techno Glass, Canada).

MORPHOLOGY AND CULTURAL DESCRIPTIONS

Because cultures may segregate into the extreme culture types (Hansen 1938) and not be representative of the 'average' culture, the use of single-spore cultures is not recommended for characterisation and classification of *Phoma* spp. collected from fields (Boerema *et al.* 2004). Hence single spore isolates were not used in this assessment of cultural and morphological variability.

All isolates were refreshed by placing stored agar pieces (from -80°C) onto PDA and incubating in darkness at 20 to 22°C for one week. Isolates were then subcultured in triplicate onto oatmeal agar (OA) and malt-extract agar (MA) for mycelial growth measurement and description of cultural characteristics. For OA, 40 g rolled oats was boiled in 1L distilled water for five min and then strained through cheesecloth. The resultant liquid was made up to 1L with distilled water before adding 15 g agar no.1 and autoclaving. MA was made of 40 g malt extract-oxoid L39, 15 g oxoid agar no.1 and 1L distilled water. Petri plates of MA and OA were inoculated with a 5 mm diameter mycelial plug taken from the actively growing margin of cultures on PDA. Inoculated plates were incubated in darkness at 20°C for seven days and then placed in an incubator for a further seven days at 20 to 22°C with a day-night regime of 14 h under an 18W cool daylight fluorescent globe (TL-D18W/1865, Phillips) and 10 h darkness to stimulate sporulation and pigment production.

Colony diameter was measured for each isolate on both media after seven days. The cultural appearance of each isolate was described after 14 days using the

method of Boerema *et al.* (2004). The colonies were assessed by eye for regularity of colony margin; mycelial colour, aerial mycelia density; and development of pycnidia. A mycological colour chart was used for colour description of colonies (Rayner 1970). Conidial dimensions were recorded for cultures that sporulated on OA. Individual pycnidium were squashed onto slides and stained with aniline blue (0.1% w/v). Measurements were made at 400 × magnification with a calibrated graticule with a Zeiss Axioskop 2 plus microscope fitted with Zeiss plan-neofluar objective lenses. For each fungal isolate replicate, 20 randomly selected conidia were measured in at least 10 different microscopic fields when sufficient conidia were found. All conidia were measured when less than 20 were found.

TYPING TO VARIETY

Each isolate was tested for the presence of antibiotic metabolite 'E' after 14 days. A drop of 5M sodium hydroxide (NaOH) was applied to agar near the growing margin of cultures grown on malt-extract agar (MA). Production of a greenish spot or ring after 10 to 15 min, which oxidised to red pigment after approximately 30 to 60 min, indicated the presence of metabolite 'E' and a positive reaction (Boerema *et al.* 2004; Logan and O'Neil 1970).

DATA ANALYSIS

Statistical analysis of mycelial growth rate and conidial dimensions of the two Tasmanian *P. ligulicola* populations was done using analysis of variance (ANOVA) with the software programme GENSTAT version 9.1 (VSN International). Mycelial growth was analysed separately for the two populations on OA and MA for significant differences ($P < 0.05$) among isolates and to test for an interaction between isolates and media. The two populations were also compared for differences in growth on each agar medium. Conidium lengths, widths and length:width ratios were analysed separately for significant differences among isolates and between the two *P. ligulicola* populations.

RESULTS

FUNGAL ISOLATIONS

Isolations from diseased stems for the within-field population yielded 58 *P. ligulicola* isolates and two *P. exigua* isolates. Isolations from the three within-field plots in October 2006 yielded 55 *P. ligulicola* isolates from 150 diseased stems. Sampling from these same plots in December 2006 produced only a further three *P. ligulicola* isolates from 150 diseased stems.

Other fungal species isolated, but not used for this study, include *Microsphaeropsis tanaceti, Alternaria* spp. and *Stemphylium botryosum.*

TYPING TO VARIETY

All isolates collected from Tasmanian pyrethrum crops (within and between-field populations) showed a negative reaction with the sodium hydroxide test (Figure 3.1) and were designated as *P. ligulicola* var. *inoxydabilis.* By contrast, the five foreign isolates obtained from chrysanthemum showed a strong positive reaction and were identified as *P. ligulicola* var. *ligulicola* (Figure 3.2). The four *P. exigua* isolates had moderate positive reactions to the NaOH test. These isolates were identified as *P. exigua* var. *exigua* because of this result, combined with the cultural appearance (Michael Priest *pers comm*).

Figure 3.1. The Na OH spot test for production of metabolite E on MA. No colour change and negative reaction for *Phoma ligulicola* var. *inoxydabilis* isolate 62607 from pyrethrum.

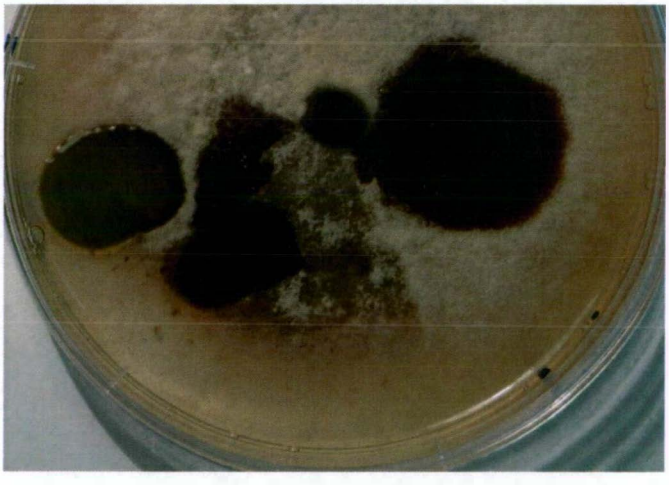

Figure 3.2 The NaOH spot test for production of metabolite E on MA. Colour change from green (after 10 min) to red (after 30 min) for German *Phoma ligulicola* var. *ligulicola* isolate DZSM 62547 from chrysanthemum.

MORPHOLOGY AND CULTURAL DESCRIPTIONS

Between-field population

Mycelial growth of the 58 *P. ligulicola* var. *inoxydabilis* isolates ranged from 35 to 67 mm after seven days on OA and 35 to 67 mm on MA. There was a significant difference ($P < 0.001$) between isolates and a significant interaction between isolate and medium ($P < 0.001$). Cultural morphology varied on OA with surface mycelial colour varying from colourless through to dull green, greenish olivaceous and grey olivaceous. Colony outlines were regular to slightly irregular and some isolates had concentrically zonate rings near the margin (Figures 3.3 A and B). Aerial mycelium was white and varied from felty to floccose, and from sparse to dense. The variation in each cultural characteristic was continuous; hence the isolates could not be classified into distinct morphological groups. Colonies on MA had white to pale olivaceous aerial mycelium, sometimes with pale buff patches (Figures 3.4 A and B). Aerial mycelia texture varied from felty to woolly and was generally dense over the entire colony. Colony outlines varied from regular to crenate. Several isolates also had zonation (Figure 3.3 A). The reverse –side of the colonies on MA was generally pale luteous with olivaceous centre, sometimes with hazel rings near the colony margin.

Perithecia were not observed on any cultures after 14 days. Nine of the 58 within-field population isolates failed to produce pycnidia, while pycnidia production on of the five cultures was rare and the pycnidia that were found contained relatively few conidia. Pycnidia varied in colour from honey to brown. This colour variation was apparent between isolates and sometimes between pycnidia of a single culture. Pycnidia were superficial or partly submerged in the agar. Production of pycnidia ranged from rare to abundant and the colourless cultures produced notably less pycnidia than those with green to olivaceous mycelium. Conidia were hyaline and aseptate, ellipsoidal to oblong. Conidial dimensions ranged from 2.5 to 10.0 µm × 1.8 to 3.8 µm. Conidia length:width ratio ranged from 1.6 to 2.3, with an average length:width ratio of

2.0. Length and width dimensions were significantly different among the isolates ($P < 0.001$).

Within-field population

Colony growth after seven days for the 58 isolates ranged from 29 to 62 mm on OA and 31 to 67 mm diameter on MA. Growth rates were significantly different among the isolates ($P < 0.001$) and there was a significant interaction with media ($P < 0.001$). Cultural characteristics were similar to those of the between-field population with differences in colony outline, colony colour and aerial mycelia colour and texture on both OA and MA, but no distinct morphological groupings. Zonate rings were apparent on several isolates on both OA and MA. Pycnidia production for these isolates was similar to those described in the between-field population and no perithecia were detected. Fifty of the 58 isolates produced pycnidia. Conidia were aseptate, ellipsoidal to oblong. Length and width of conidial were significantly different among the isolates ($P < 0.001$). Dimensions ranged from 2.5 to10 µm × 1.8 to 3.8 µm and the length:width ratio ranged from 1.5 to 2.2, with an average length:width ratio = 1.9. This population of isolates was collected from three relatively small, but separated locations within a single field. Cultural variability was distributed throughout the three plots with no cultural type coming only from a single plot. Similarly isolates from each of the three plots displayed variability in growth rates and conidial dimensions.

In comparisons of the two *P. ligulicola* var. *ligulicola* populations, conidia of between-field population were significantly wider ($P < 0.001$) while within-field population conidia were significantly longer ($P < 0.001$). This was evident in the larger length:width ratios found for the two populations (Table 3.1). The between-field population had significantly faster mycelial growth on MA ($P < 0.001$) and the two populations had similar growth on OA (Table 3.1).

Foreign isolates

Colonies on OA had regular outlines and reached 49 to 67 mm diameter after seven days. On MA colonies were 47 to 62 mm diameter after seven days and also had regular outlines.

Colony surface colours on OA varied from olivaceous or greenish olivaceous, or the entire culture was colourless with grey olivaceous centres. Aerial mycelium was white, sparse and floccose (Figure 3.3 C). Colonies on MA had regular outlines and dense felty to floccose, sometimes patchy, pale olivaceous grey or white aerial mycelium over most of the colony (Figure 3.4 C). Two isolates had zonate rings near the colony margin on both OA and MA. The reverse side of colonies had a grey olivaceous centre and the luteous pigmentation of the agar was evident for cultures on MA. The American isolate (ATCC 10748) differed from the other four isolates from Germany (DSMZ 10673 and 62547) and New Zealand (ICMP 2287 and 10673) in that the aerial mycelia was felty, not floccose, on MA (Figure 3.4 C).

Isolates from Germany and New Zealand produced abundant honey, brown or black coloured pycnidia, superficial and submerged in the agar, which were distributed over the colony on OA. The American isolate (ATCC 10748) produced notably less and very small pycnidia which were submerged in the agar. Conidia were hyaline, ellipsoidal to oblong, mostly aseptate, with some being 1-septate. Conidia dimensions ranged from 2.5 to 12.5 × 1.9 to 3.8 µm and length:width ratio ranged from 1.5 to 2.6. Isolate ATCC10748 had the smallest conidia of all three *P. ligulicola* populations. No perithecia were detected after 14 days.

Table 3.1. Mean mycelial growth (diameter) and standard deviation after 7 days, and conidial dimensions and standard deviations after 14 days.

	Mycelial growth (mm)		Conidial dimensions (µm)		
Population	MA	OA	Length	Width	Length:width ratio
Between-field[1]	49.6 ± 8.8	47.4 ± 7.4	5.0 ± 1.1	2.5 ± 0.1	2.0 ± 0.5
Within-field [2]	46.2 ± 9.9	47.1 ± 6.3	4.9 ± 1.0	2.5 ± 0.2	1.9 ± 0.4
Foreign[3] (*n* = 5)	56.5 ± 5.9	57.7 ± 7.7	5.4 ± 2.0	2.5 ± 0.3	2.1 ± 0.7
Phoma exigua[4] (*n* = 4)	50.3 ± 18.9	66.8 ± 19.2	4.7 ± 1.0	2.5 ± 0.1	1.9 ± 0.4

[1] *Phoma ligulicola* var. *inoxydabilis* population obtained from pyrethrum crops on a regional scale (*n* = 58)

[2] *Phoma ligulicola* var. *inoxydabilis* population obtained from one pyrethrum crop (*n* = 58*)*

[3] *Phoma ligulicola* var. *ligulicola* population from Germany (*n*=2), New Zealand (*n*=2) and USA (*n*=1)

[4] *Phoma exigua* population from pyrethrum crops on a regional scale (*n*=2) and obtained from one pyrethrum crop (*n*=2)

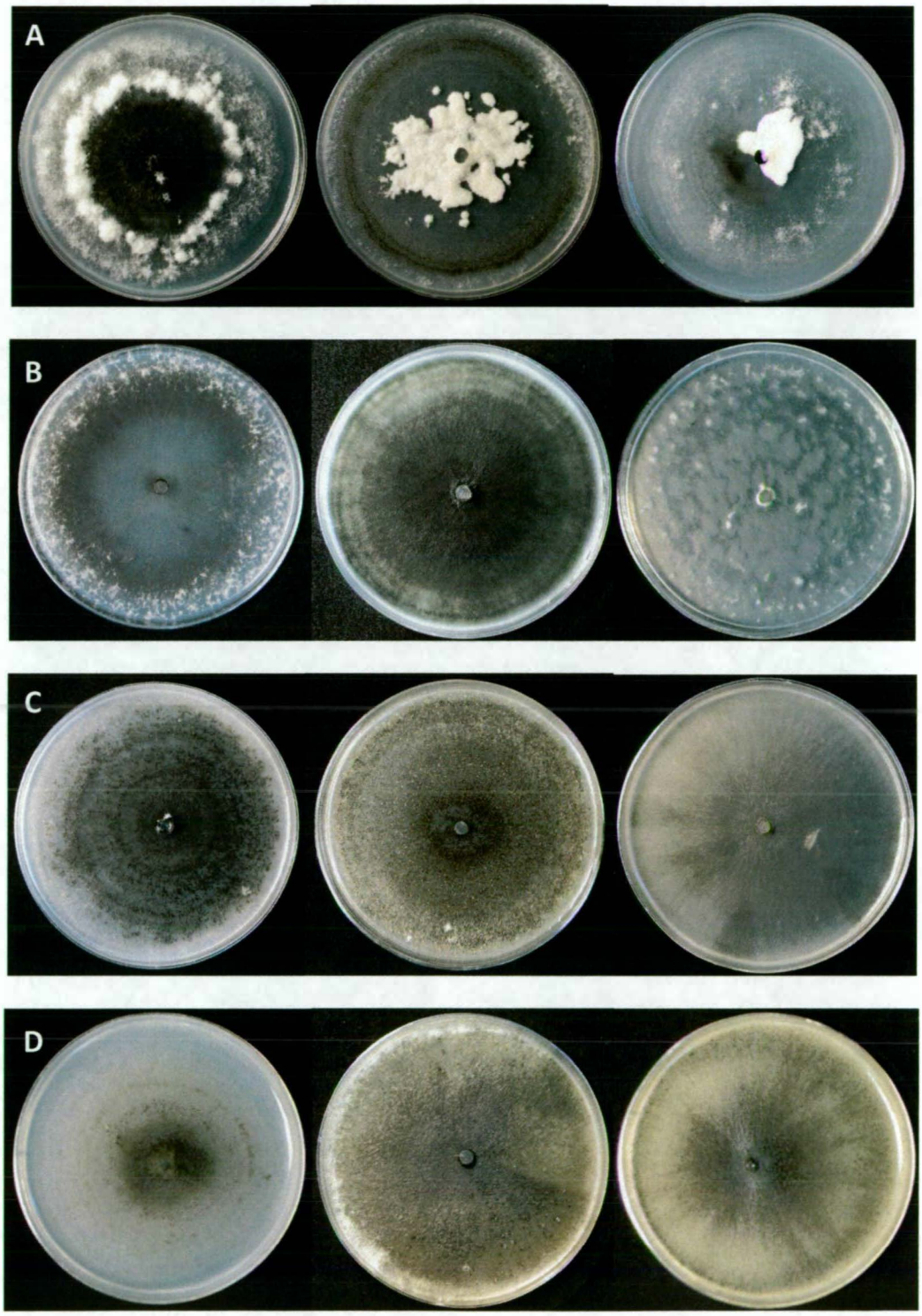

Figure 3.3. *Phoma* cultures on oatmeal agar. (A) and (B) *Phoma. ligulicola* var. *inoxydabilis* from pyrethrum. (C) *Phoma ligulicola* var. *ligulicola* from chrysanthemum. (D) *Phoma exigua* var. *exigua* from pyrethrum.

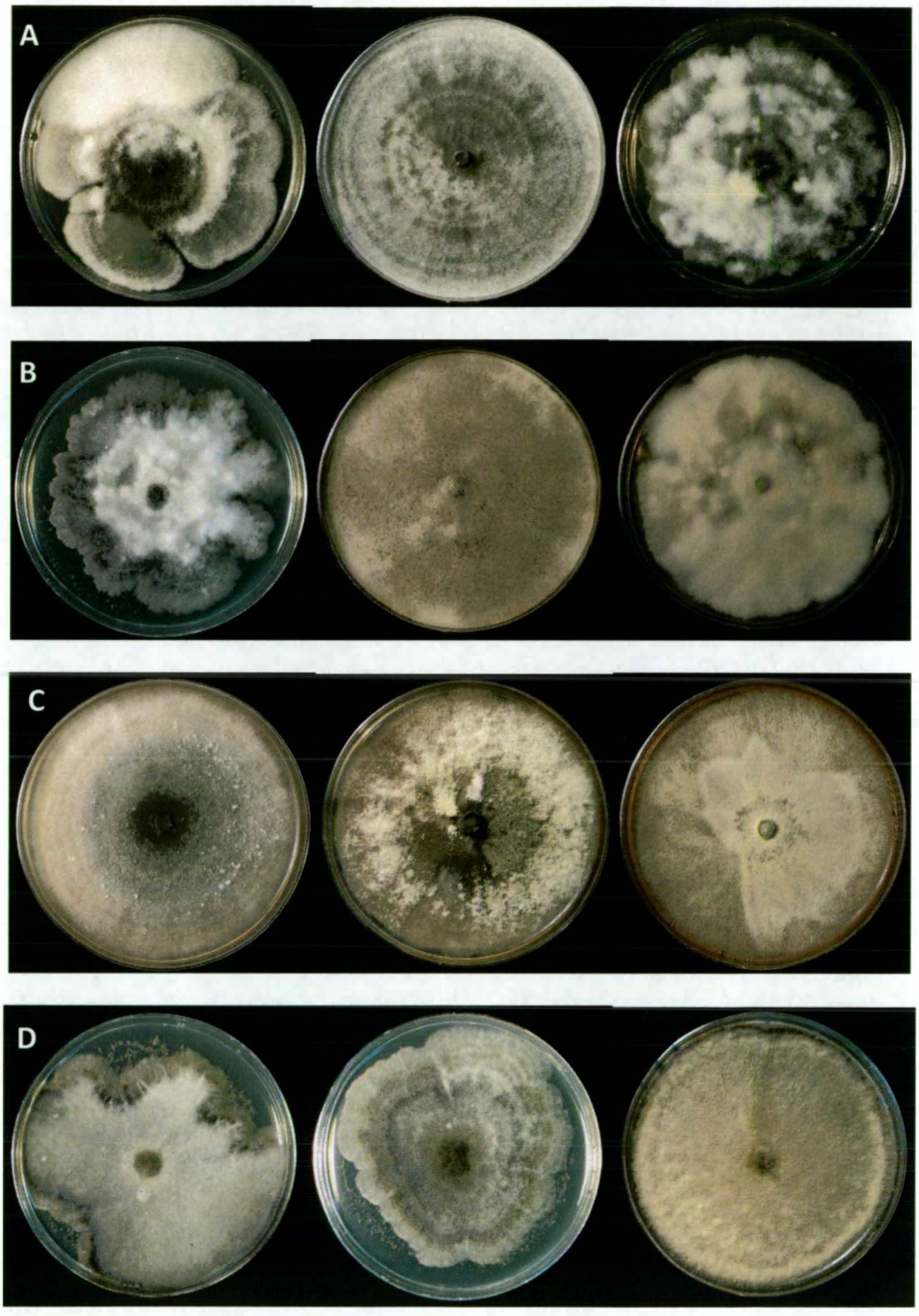

Figure 3.4. *Phoma* cultures on malt extract agar. (A) and (B) *Phoma ligulicola* var. *inoxydabilis* from pyrethrum. (C) *Phoma ligulicola* var. *ligulicola* from chrysanthemum. (D) *Phoma exigua* var. *exigua* from pyrethrum.

Phoma exigua

On OA colonies had regular to irregular outlines and mycelial growth varied from 41 to 84 mm after seven days. Growth on MA ranged from 30 to 70 mm and colony margins were crenate or regular, and sometimes both. Culture colours on OA varied from greenish olivaceous to olivaceous or olivaceous grey sometimes with a patchy radiating pattern and some cultures were colourless near the margin (Figure 3.3 D). Aerial mycelium was sparse, white and floccose. On MA, cultures varied in colour, sometimes with combinations of olivaceous, olivaceous buff and olivaceous grey felty dense aerial mycelium (Figure 3.4 D). Undersides of colonies on MA had an olivaceous black centre with colourless and honey patches near the margins.

All four isolates produced moderate to abundant brown pycnidia, solitary or aggregate, partially submerged in agar and were distributed over most of the colony. Conidia were hyaline ellipsoidal to oblong, mainly aseptate, but occasionally 1-septate with dimensions ranging from 2.5 to 7.5 µm × 1.8 to 3.75 µm (length:width ratio = 1.7 to 2.1).

DISCUSSION

While the isolates were within the context of published descriptions, this study found variation in the morphological and cultural characteristics for the 116 *P. ligulicola* var. *inoxydabilis* isolates collected from Tasmanian pyrethrum crops. Cultural variability was evident within the two populations, and within the three sub-groups of the between-field population. Cultural variability was continuous across the Tasmanian *P. ligulicola* isolate collection and there was no clear distinction of cultural 'types' for either population. While cultural appearance also varied among the five foreign *P. ligulicola* var. *ligulicola* isolates, there was no clear distinction between isolates from New Zealand and Germany. The American isolate differed from the other foreign isolates in cultural appearance on MA and on OA had conspicuously fewer and smaller pycnidia and conidia.

A number of Tasmanian cultures had zonate rings, a phenomenon which is reported to occur only on *P. ligulicola* var. *ligulicola* cultures (Boerema *et al.* 2004). Hence this characteristic could not be used to differentiate the two *P. ligulicola* varieties. However the NaOH spot test for production of metabolite E clearly distinguished the two *P. ligulicola* varieties. The foreign isolates produced notably less aerial mycelia than most of the Tasmanian isolates on OA. Logan and O'Neil (1970) reported a similar phenomenon for strains of *P. exigua* var. *foveata*. Strains that produced metabolite E produced little or no aerial hyphae on MA compared to E negative cultures which produced abundant aerial hypha. The *P. exigua* cultures were clearly distinguishable from *P. ligulicola* isolates on MA, by the aerial hyphae colour and highly irregular colony margin. But on OA the *P. exigua* isolates were similar to some of the *P. ligulicola* isolates, in colony colour and negligible aerial mycelium production. These findings highlight the need to inspect fungal cultures on a variety of media to detect differences in cultural morphology among species and varieties and to enable correct identification.

Mycelial growth rates were variable among isolates for the two Tasmanian *Phoma ligulicola* populations and the foreign isolates. The range of growth rates for the between-field and within-field populations on OA were similar, but showed greater variability on MA. The range of growth rates for within-field population indicates that this variability is natural for *P. ligulicola* var. *inoxydabilis* and is not due to phenotypic divergence of geographically separated populations. Both populations contained isolates with faster and slower growth than previously published reports for *P. ligulicola* var. *inoxydabilis* on both media. Based on published records, mycelial growth rates for *P. ligulicola* var. *inoxydabilis* range from 45 to 50 mm on OA and 35 to 40 mm on MA (de Gruyter *et al.* 2002). In the present study, growth rates for *P. ligulicola* var. *inoxydabilis* isolates varied significantly and ranged from 29 to 67 mm on OA and from 31 to 67 mm on MA. The percentage of isolates with growth rates outside the published records were 52 and 86 % for OA and MA respectively. Conversely,

growth rates on OA recorded for the five foreign *P. ligulicola* var. *ligulicola* isolates were generally slower than the 70 mm recorded by de Gruyter *et al* (2002), but were close to the range of 50 to 60 mm growth on MA after seven days. Several Tasmanian isolates had growth rates that were equal to the reportedly faster growing *P. ligulicola* var. *ligulicola* isolates (de Gruyter *et al.* 2002). As such, growth rates could not be reliably used as criteria for distinguishing between these two varieties in this study.

Morphological criteria used to differentiate *P. exigua* varieties overlap or are common for several varieties (Aa van der *et al.* 2000). This makes conclusive identification difficult, especially when phenotypic differences may occur due to geographic and host-interaction relationships. Published reports of *P. exigua* var. *exigua* mycelial growth rates are highly variable (2.5 to 8.5 mm on OA and 2.5 to 7.5 mm on MA after 7 days) (Aa van der *et al.* 2000). With such a wide range, it was not surprising that the growth rates recorded for the Tasmanian *P. exigua* isolates were consistent with published records. Hence this information is of limited use for classification of this species and variety. The four isolates were culturally different to *P. ligulicola* var. *inoxydabilis* and similar to descriptions of *P. exigua* var. *exigua.*

The positive result for the NaOH test combined with the morphological and cultural descriptions indicate that the four *P. exigua* isolates in this study are *P. exigua* var. *exigua*. A macrochemical reaction, the sodium hydroxide (NaOH) spot test for production of antibiotic metabolite 'E' (derived from *exigua*), is used in conjunction with morphological examination to differentiate varieties of *P. exigua* (Logan and O'Neil 1970). Two varieties of *P. exigua*, var. *exigua* and var. *linicola* produce antibiotic E, and some strains of var. *heteromorpha* also show a mild positive reaction to the NaOH test (Aa van der *et al.* 2000). The latter two *P. exigua* varieties are specific pathogens of flax and oleander respectively, and not likely to occur on pyrethrum in Tasmania.

Conidial dimensions were significantly different among isolates and ranged from 2.5 to10 µm long and 1.9 to 3.8 µm wide for the two Tasmanian populations. For the between-field population, 93.3% of the conidia lengths and 99.7% of the conidia widths were within the range reported for *P. ligulicola* var. *inoxydabilis* of 3.5 to 13 × 2 to 4 µm. For the within-field population, 91.2% of conidium lengths and 99.3% conidium widths were within the reported range. For the foreign isolates, 89.3% of conidium lengths and 90.7% of conidium widths were within published dimensions for *P. ligulicola* var. *ligulicola* of 3.5 to 12 µm long and 2 to 4 µm wide. The finding that conidia of *P. ligulicola* var. *ligulicola* were larger than *P. ligulicola* var. *inoxydabilis* agrees with the results of de Gruyter *et al.* (2002). However, the American *P. ligulicola* var. *ligulicola* isolate was the exception in this case as it produced conidia that were similar in size to the smallest recorded for *P. ligulicola* var. *inoxydabilis* in this study. For both *P. ligulicola* varieties, conidial dimensions outside the published dimensions were always smaller than the recorded range. The smaller conidia detected in this study may be explained by minor differences in the experimental conditions of this study and those of published studies. Conidia produced at higher temperatures tend to be smaller than those produced at lower temperatures (Blakeman and Hadley 1968) and a temperature variation of 2°C is sufficient to make a measurable difference (McCoy and Dimock 1972).

The isolates in this study also varied in the abundance of pycnidia and how pycnidia were distributed throughout the colony. Little information is available regarding the distribution of pycnidia on colonies but an early study found that *P. ligulicola* var. *ligulicola* produced mature pycnidia only in the centre of colonies (Baker *et al.* 1949). The current study found that colonies of *P. ligulicola* var. *ligulicola* had abundant mature pycnidia distributed over most areas of the colony, while for *P. ligulicola* var. *inoxydabilis*, mature pycnidia were generally concentrated either in the centre of the colony or near the margin. Pycnidial production of *P. ligulicola* can be sporadic among isolates (Baker *et al.* 1949; McCoy 1971). This phenomenon was reported in a previous study of

P. ligulicola var. *inoxydabilis* from pyrethrum (Pethybridge *et al.* 2005c) and was also evident in the current study. Hence these key morphological features could not be examined for the seventeen *P. ligulicola* var. *inoxydabilis* isolates that did not sporulate.

Perithecia were not found for the five foreign *P. ligulicola* var. *ligulicola* isolates, which agrees with published information (Aa van der *et al.* 1990; de Gruyter *et al.* 2002). The methods used in my study were reported to be conducive for *in vitro* production of mature perithecia by *P. ligulicola* var. *inoxydabilis* (Aa van der *et al.* 1990; de Gruyter *et al.* 2002). However, none were detected for the 116 isolates that were examined in culture. Loss of the ability to form perithecia through culture storage and repeated subculturing has been observed in other ascomycetes (Furukawa and Kishi 2002). This could explain why the between-field population did not produce perithecia, as these isolates were 2 to 4 years old and are likely to have been subcultured repeatedly. However, the within-field isolates were collected eight weeks before the cultural morphology assessment was done, and they were only subcultured once since isolation. The failure of *P. ligulicola* var. *inoxydabilis* to produce perithecia meant that an important morphological could not be observed in this study.

While the findings of this study generally agreed with published reports and cultural descriptions, the variability and overlap in morphology and cultural characteristics observed in this study made conclusive differentiation between the two *P. ligulicola* varieties difficult. Additionally, there was some divergence between the observations of this study and the published descriptions of *P. ligulicola*. These discrepancies emphasize the problems associated with identification of newly acquired *P. ligulicola* isolates from the field and highlight the need to use a suite of complementary tools to classify and assess fungal pathogen species which are not morphologically distinct.

CHAPTER 4

GENETIC VARIABILITY OF *PHOMA* SPECIES FROM PYRETHRUM AND CHYRYSANTHEMUM

INTRODUCTION

Problems with the accurate identification of *Phoma* species based on morphological and biological characteristics have been discussed in Chapter 3. DNA based molecular techniques can supplement traditional fungal identification methods and may also provide information about the pathogens evolution and ancestral relationships. There are many genomic regions available for phylogenetic studies and the level of taxonomic resolution that can be achieved depends on several elements, including how well conserved the target region is and the rate that mutations accumulate in a particular region. These factors vary with species and there are no standard delimiters for the distinction of taxa at particular levels. Hence gene regions are chosen according to the type of information that is required. Thresholds of sequence identity for distinction of species vary depending on the genus and on the genetic region of interest. For example, two species of *Pyrenophora* (*P. graminea* and *P. teres*) are differentiated by just one base substitution in the ITS region (Stevens *et al.* 1998) while sequences of glyceraldehyde-3-phosphate dehydrogenase (G3PD) for two isolates of *Saccharomyces cerevisia* were found to vary at 11% of nucleotide sites (Bruns *et al.* 1991). A single genomic region may be all that is necessary to confirm the identity of a pathogen or for detection purposes. However a good representation of the phylogeny of an organism generally requires comparison of several, preferably physically and functionally distinct regions (Bruns *et al.* 1991). Hence, studies of fungal populations frequently examine multiple genomic regions to gain a more comprehensive representation of genetic variability.

INTERNAL TRANSCRIBED SPACER

The internal transcribed spacer (ITS) regions (ITS1 and ITS2) and the 5.8S rRNA gene are commonly used for determining relationships between genera and species (Abeln *et al.* 2002; Glass and Donaldson 1995; Peever *et al.* 2007; White *et al.* 1990). ITS regions are less conserved than other regions, such as the protein-coding regions, and can show variability at an intraspecific level (Arabi and Jawhar 2007). Additionally they are present in multiple copies, within regions that are highly conserved, which improves sensitivity of the PCR assay (White *et al.* 1990). The nuclear ribosomal DNA (rDNA) which contains tandem repeats of three rRNA genes (18S, 5.8S and 28S) and ITS regions are widely used in phylogenetic and population studies (Arabi and Jawhar 2007; Arenal *et al.* 2000; Balmas *et al.* 2005; Camara *et al.* 2002; Glass and Donaldson 1995; Lindqvist-Kreuze *et al.* 2003; Somai *et al.* 2002). ITS1 and ITS2 regions are located either side of the 5.8S gene, and flanked by the 18S and 28S molecules. The noncoding ITS regions are highly variable in length and sequence composition. By comparison, rRNA genes contain both highly conserved and variable sequences that do not vary greatly in length (Kennedy and Clipson 2003). Because rRNA genes evolve slowly compared to the ITS regions they are useful targets for universal primers and for long-term evolutionary studies, while the relatively variable ITS regions are more informative of recent genetic divergence and differences among genera and species (White *et al.* 1990).

GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE

The nuclear gene that encodes the enzyme, glyceraldehyde-3-phospate dehydrogenase (G3PD), is also commonly used for species identification and phylogenetic studies. The enzyme is involved in glycolysis, the metabolic pathway that enables organisms to break down glucose. The G3PD protein coding gene is highly conserved, but the third codon positions and introns are

variable enough to be phylogenetically informative at interspecific, and in some cases intraspecific levels (Myllys *et al.* 2002; Poggeler 1999). This region has been used for multigene comparisons of various fungi (Camara *et al.* 2002; Olvera *et al.* 2006).

TRANSLATION ELONGATION FACTOR

The translation elongation factor (EF1-α) gene is a highly conserved protein encoding gene that can also reveal genetic variability at both inter- and intraspecific levels (Carbone *et al.* 1999; Rehner and Buckley 2005). This gene is involved in the translation (protein synthesis) process in eukaryotes (Slobin 1980) and the protein carries aminoacyl-tRNA to ribosomes (Russel 1994). EF1-α exons are highly conserved and they can be informative even at low level phylogenetics because the third position exons evolve at similar rates to introns (Rehner and Buckley 2005). Introns are non-coding regions within protein coding genes and are generally less conserved and more variable than the coding regions (Glass and Donaldson 1995). The EF1-α region has been used alone for phylogenetic studies of fungal species (Maphosa *et al.* 2006) and also to supplement data derived from other regions such as the ITS and G3PD (Crous *et al.* 2004; Jacobs *et al.* 2005; Peever *et al.* 2007).

To the best of my knowledge, there are no published reports on the use of DNA sequence data from protein-coding genes for identification or phylogenetic analysis of *P. ligulicola*. This chapter examines genetic variability of Tasmanian *P. ligulicola* var. *inoxydabilis* isolates obtained from pyrethrum. ITS, EF1-α and G3PD sequences were used to assess intraspecific and interspecific genetic relationships of *P. ligulicola* and *P. exigua* isolates. A representative group of isolates were selected for direct sequencing, comparison of aligned sequences and analysis of phylogenetic relationships with closely related species. The five *P. ligulicola* var. *ligulicola* isolates from chrysanthemum and the four *P. exigua* isolates used in the morphological study, discussed the previous chapter, were also included in this study.

The aims of this study were to:

1. Use phylogenetic analysis to confirm the identity of *Phoma* species isolates obtained from pyrethrum crops in Tasmania and evaluate their genetic relatedness to closely related species.
2. Evaluate within-species genetic variability of Tasmanian *Phoma* spp. isolates from pyrethrum based on three genomic regions, ITS region (ITS1, 5.8s gene and ITS2), G3PD, and EF1-α.
3. Compare the sequences of *P. ligulicola* var. *inoxydabilis* isolates collected from pyrethrum crops in Tasmania with *P. ligulicola* var. *ligulicola* isolates sourced from chrysanthemum ray blight in USA, New Zealand and Germany.

MATERIALS AND METHODS

ISOLATES USED IN THIS STUDY

A representative group of isolates were selected from those examined in the previous chapter. These were chosen on the basis of morphological variability and prior studies of genetic variability (Pethybridge *et al.* 2005c). The majority of Tasmanian *Phoma* spp. used in this study were from the between-field population (outlined in Chapter 2) and as such were geographically and temporally separated individuals. It was anticipated this would provide the broadest and therefore most representative population. The five *P. ligulicola* var. *ligulicola* isolates sourced from various international culture collections were included for comparison. The four *P. exigua* var. *exigua* isolates obtained from pyrethrum were also included for comparison with *P. ligulicola* sequences and for further confirmation of species identification. The isolates used in this study are outlined on Table 4.1.

Table 4.1. Isolates of *Phoma ligulicola* and *Phoma exigua* or fungal sequences used for phylogenetic analysis of the translation elongation factor (EF1-α), internal transcribed spacer (ITS) and glyceraldehyde-3-phosphate dehydrogenase (G3PD) regions.

Isolate Code	Anamorph	Teleomorph	Geographic origin	Host	EF1-α Accession	ITS Accession	G3PD Accession
PL1	*P. ligulicola* var. *inoxydablis*	*D. ligulicola*	Tasmania	*T. cinerariifolium*	-	-	-
PL3	*P. ligulicola* var. *inoxydabilis*	*D. ligulicola*	Tasmania	*T. cinerariifolium*	-	AY157877	-
PL4	*P. ligulicola* var. *inoxydabilis*	*D. ligulicola*	Tasmania	*T. cinerariifolium*	-	-	-
PL7	*P. ligulicola* var. *inoxydabilis*	*D. ligulicola*	Tasmania	*T. cinerariifolium*	-	AY157882	-
PL12	*P. ligulicola* var. *inoxydabilis*	*D. ligulicola*	Tasmania	*T. cinerariifolium*	-	-	-
PL15	*P. ligulicola* var. *inoxydablis*	*D. ligulicola*	Tasmania	*T. cinerariifolium*	-	AY157876	-
PL46009	*P. ligulicola* var. *inoxydabilis*	*D. ligulicola*	Tasmania	*T. cinerariifolium*	-	-	-
PL50904	*P. ligulicola* var. *inoxydabilis*	*D. ligulicola*	Tasmania	*T. cinerariifolium*	-	-	-
PL52301	*P. ligulicola* var. *inoxydabilis*	*D. ligulicola*	Tasmania	*T. cinerariifolium*	-	-	-
PL58905	*P. ligulicola* var. *inoxydablis*	*D. ligulicola*	Tasmania	*T. cinerariifolium*	-	-	-
PL58906	*P. ligulicola* var. *inoxydabilis*	*D. ligulicola*	Tasmania	*T. cinerariifolium*	-	-	-
PL62704	*P. ligulicola* var. *inoxydabilis*	*D. ligulicola*	Tasmania	*T. cinerariifolium*	-	-	-
PL71401	*P. ligulicola* var. *inoxydablis*	*D. ligulicola*	Tasmania	*T. cinerariifolium*	-	-	-
DAR70020	*P. ligulicola* var. *inoxydabilis*	*D. ligulicola*	Tasmania	*T. cinerariifolium*	-	AY157887	-
ATCC10748	*P. ligulicola* var. *ligulicola P.*	*D. ligulicola*	USA	*C. morifolium*	-	-	-
DSMZ62457	*ligulicola* var. *ligulicola P.*	*D. ligulicola*	Germany	*C. morifolium*	-	AY157889	-
DSMZ63133	*ligulicola* var. *ligulicola P.*	*D. ligulicola*	Germany	*C. morifolium*	-	AY157888	-
ICMP2287	*ligulicola* var. *ligulicola*	*D. ligulicola*	New Zealand	*C. morifolium*	-	-	-
ICMP10673	*P. ligulicola* var. *ligulicola*	*D. ligulicola*	New Zealand	*C. morifolium*	-	-	
PE63210	*P. exigua* var. *exigua*	-	Tasmania	*T. cinerariifolium*	-	-	-
PEMRA	*P. exigua* var. *exigua*	-	Tasmania	*T. cinerariifolium*	-	-	-
PE8-25	*P. exigua* var. *exigua*	-	Tasmania	*T. cinerariifolium*	-	-	-
PE8-26	*P. exigua* var. *exigua*	-	Tasmania	*T. cinerariifolium*	-	-	-

Isolate Code	Anamorph	Teleomorph	Geographic origin	Host	EF1-α Accession	ITS Accession	G3PD Accession
-	*Asochyta fabae*	*Didymella fabae*	USA	*Vicia faba*	DQ386492	DQ383952	DQ383959
-	*Ascochyta lentis*	*Didymella lentis*	Australia	*Lens culinaris*	DQ386493	DQ383953	DQ383961
-	*Ascochyta pinodes*	*Mycosphaerella pinodes*	USA	*Pisum sativum*	-	DQ383957	DQ383975
-	*Phoma cucurbitacearum*	*Didymella bryoniae*	-	-	-	AB266850	-
-	*P. exigua*	-	-	-	AY831544	-	-
-	*P. exigua*	-	-	-	-	-	
-	*Phoma glomerata*	-	USA	*Platanus occidentalis*	-	AF126816	-
-	*Phoma herbarum*	-	Australia	-	-	AF218792	-
-	*Phoma medicaginis*	-	USA	*Medicago sativa*	EU394715		
-	*P. medicaginis*	-	-	-		EU167575	-
-	*P. medicaginis*	-	-	*M. sativa*		--	DQ525740
-	*Phoma pinodella*	*Ascochyta pinodella*	USA	*Cicer arietinum*	-	-	DQ383979
-	*P. pinodella*	*A. pinodella*	-	-		EU167565	-
-	*P. pinodella*	*A. pinodella*	Australia	*Medicago truncatula-*	AY831542		
-	*Phoma sclerotiodes*	-	-	*M. sativa*	-	-	DQ525737

DNA EXTRACTION

Mycelium was scraped from the top of 14 day old cultures growing on PDA and added to 20 ml of 2% malt-extract broth in petri dishes sealed with electrical tape. Broth mixtures were incubated at approximately 20°C under constant illumination by fluorescent lights and agitated on an orbital shaker at 50 rpm. After 10 to 14 days mycelium (0.2 - 1 g) was removed from the broth and excess moisture was blotted from the fungal tissue using paper towels. DNA extraction from the mycelium was completed using the DNeasy Plant Mini Kit (Qiagen, GmbH, D40724, Hilden Germany) following manufacturer's instructions (Appendix 1).

DNA AMPLIFICATION

Translation elongation factor 1- alpha (EF1-α)

Fourteen *P. ligulicola* var. *inoxydabilis* isolates and four *P. exigua* var. *exigua* isolates (Table 4.1) collected from Tasmanian pyrethrum crops were selected for sequencing and phylogenetic analysis of the EF1-α genomic region. Five *P.* ligulicola var. ligulicola isolates sourced from various international culture collections were also used for this study (Table 4.1).

Primers EFCF1 (AGTGCGGTGGTATCGACAAG) and EFCF6 (CATGTCACGGACGGCGAAAC) (Tom Harrington, Iowa State University, *unpublished data*) were used to amplify a ~1200 bp fragment of the EF1-α region. These primers and PCR reaction mixtures and cycling conditions were based on the method used by Rehner and Buckley (2005). PCR conditions were optimised and amplifications were performed in a total reaction volume of 50 µl. The PCR reaction mixture consisted of 1 × Qiagen PCR buffer with 1.5 mM $MgCl_2$ (Applied Biosystems Inc., Foster City, CA), 200 µM dNTPs, 0.4 µM each primer, 1 unit *Taq* polymerase and 20 to 40 ng DNA. All PCR and sequencing reactions were performed on a GeneAmp PCR Systems 2400 thermocycler (Perkin Elmer, Norwalk, CT, USA). Cycling conditions consisted of initial denaturation for 5 min

at 96°C, followed by six touch down cycles of 94°C denaturation for 35 s, 1 min annealing at 68°C to 63°C with a 1°C drop per cycle, and a 2 min extension at 72°C. This was followed by a further 29 cycles of 35 s denaturation at 94°C , 1 min annealing at 63°C, 2 min extension at 72°C, and a final 8 min extension step at 72°C to complete the reaction. PCR was run as hot start and reactions were transferred from ice to thermocycler when the plate temperature was at least 90°C.

Nuclear ribosomal internal transcribed spacer (ITS) and Glyceraldehyde-3-phosphate-dehydrogenase (G3PD)

A subset of six *P. ligulicola* var. *inoxydabilis* isolates with variable morphology were selected for G3PD and ITS sequencing and phylogenetic analysis (Table 4.2). The five *P. ligulicola* var. *ligulicola* and four *P. exigua* var. *exigua* isolates were also sequenced for these two regions, making a total of 15 isolates for comparison of these two regions. Four Tasmanian *P. ligulicola* var. *inoxydabilis* isolates and two *P. ligulicola* var. *ligulicola* isolates sourced from overseas had previously been sequenced for the ITS region (Pethybridge *et al.* 2005c), so the corresponding GenBank accessions were used for this study (Table 4.1). ITS sequences were generated for the three remaining *P. ligulicola* var. *ligulicola* isolates and the four *P. exigua* var. *exigua* isolates. G3PD sequences were generated for all 15 *Phoma* isolates (Table 4.2).

Universal primers ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) (White *et al.* 1990) were used to amplify approximately 540 bp of ITS1, 5.8s and ITS2 from eight isolates. Twenty -five µl PCR reactions contained 1 × PCR buffer with 1.5 mM $MgCl_2$ (Applied Biosystems Inc.), 200 µM dNTPs, 0.4 µM each primer, 1 unit *Taq* polymerase (Applied Biosystems Inc.) and 20 to 40 ng of genomic DNA. Cycling conditions consisted of: 3 min at 94°C for initial denaturation followed by 30 cycles of 30 s of denaturation at 94°C, 30 s of annealing at 60°C and 1 min extension at 72°C. Reactions concluded with a final extension at 72°C for 5 min.

Five hundred and eighty-five bp of the G3PD gene were amplified with primers gpd-1 (CAACGGCTTCGGTCGCATTG) and gpd-2 (GCCAAGCAGTTGGTTGTGC) (Berbee *et al.* 1999) from 15 isolates. G3PD reactions were carried out using the same cycling conditions and concentration of PCR reagents as those described above for sequencing the ITS region.

Table 4.2. Sequence haplotypes identified among isolates of *Phoma ligulicola* and *Phoma exigua* sampled from pyrethrum and chrysanthemum.

Haplotype	Isolates
EF1-α	
1	DAR70020, PL3, PL7, PL12, PL15, DSMZ62547, DSMZ63133 [a] (PL1, 46009, 50904, 52301, 58905, 58906, 62704, 71401)
2	PL4, ATCC10784
3	ICMP2287, ICMP10673
4	PEMRA, PE8-25,
5	PE8-26
6	PE63210
ITS	
1	DAR70020
2	PL3, PL4, PL7, PL12, PL15
3	ATCC10748, DSMZ62547, DSMZ63133, ICMP2287, ICMP10673
4	PEMRA, PE63210, PE8-25, PE8-26
G3PD	
1	DAR70020, PL3, PL4, PL7, PL12, PL15, DSMZ62547, DSMZ63133
2	ATCC10748, ICMP2287, ICMP10673
3	PEMRA, PE8-25, PE8-26
4	PE63210

[a]Sequences generated for EF1-α only
Haplotypes used for phylogenetic analysis with closely related species are underlined

DNA SEQUENCING

After PCR, 5 µl aliquots of the reactions were visualised in a 1.5% agarose (ICN Biochemicals, Ohio, USA) gel stained with ethidium bromide to confirm the presence of a single band of the correct sized product. Amplicons were then purified using a MoBio Ultraclean PCR clean-up kit (Mo Bio Laboratories, Carisbad, CA, USA). The purified DNA was quantified using a Precision Molecular Mass Standard (Bio-Rad, Laboratories, Hercules, CA, USA) and resolved on 2% agarose gel, post-stained with ethidium bromide, prior to sequencing. For sequencing of the ITS and G3PD regions, the primers used for the initial PCR were also used in the sequencing reactions.

The PCR with primers EFCF1 and EFCF6 yielded a single ~1200 bp fragment, of which only ~440 bp were sequenced with primers EFCF1 and EFCF2 (TGCTCACGGGTCTGGCCAT). Primers EFCF1, EFCF2, and EFCF6 were designed for detection of *Ceratocystis* and *Neurospora* by Tom Harrington (*unpublished data*). These three EFCF primers were developed from sequences generated from primers designed for amplification of *Fusarium oxysporum* f. sp. *cubense* (O'Donnell *et al.* 1998) and *Beauveria* and *Cordyceps* species (Rehner and Buckley 2005).

Sequencing reactions were carried out using a Dye Terminator Cycle (DTCS) Quick Start Kit (Beckman Coulter Inc., Fullerton, CA, USA). Reaction mixtures consisted of 4 µl DTCS Quick Start Master Mix, 0.16 µM forward or reverse primer and 4 µl (40 to 60 ng) DNA template in a total volume 10 µl. Thermocycler conditions consisted of a 2 min initial denaturation at 95°C, followed by 30 cycles of 20 s denaturation at 96°C, 20 s annealing at 50°C, and 4 min extension at 60°C (as per instructions in the Beckman Coulter Dye Terminator Cycle Sequencing (DTCS) Quick Start Kit (P/N 608120)). Sequencing reaction products were cleaned with an ethanol precipitation procedure according to the Beckman Coulter DTCS Kit instructions (Appendix 2).

ASSESSMENT OF GENETIC VARIABILITY AND PHYLOGENETIC ANALYSIS OF SEQUENCE DATA

Sequences generated in this study for *P. ligulicola* and *P. exigua* isolates were edited manually and aligned for comparison, identification of haplotypes, and calculation of similarities. A haplotype is defined herein as a unique combination of nucleotides in a sequence and can represent single (Karp 2008) or multilocus regions (Milgroom 1996). Haplotypes were identified separately for each locus (EF1-α, G3PD and ITS) and only unique haploypes were used for the phylogenetic analyses (Table 4.2).

For comparison to closely related species the sequences generated in this study were aligned with sequences from GenBank using AlignX (Vector NTI Advance 10.3.0, Invitrogen Corporation 2006). Maximum likelihood (ML) phylogenies were estimated independently for each data set (EF1-α, ITS and G3PD) using heuristic searches in PAUP v.4.10 (Swofford 2002). Gapped characters (insertions and deletions) were excluded from the data set during the analyses. Likelihood settings from best-fit models were selected by hierarchical likelihood ratio tests (hLRT) for the EF1-α and G3PD datasets, and by Akaike information criterion (AIC) for the ITS dataset, in MrModeltest v2.2 (Nylander 2004).

For the EF1-α dataset, the HKY+G with unequal base frequencies (A=0.2018, C=0.3071, G=0.2054, T=0.2857), transition/transversion on ratio of 1.1579 and gamma distributed rates (shape parameter = 1.5152). The final data matrix had 10 taxa and 188 characters. For the ITS dataset, which had 14 taxa and 420 characters, the SYM+I model with equal base frequencies, six substitution parameters (10.020, 4.972, 6.519, 1.193, 30.480, 1.000), transition/transversion ratio of 0.863 and equal distribution of rates at variable sites. The G3PD dataset had 10 taxa and 340 characters. The HKY+G model was selected with unequal base frequencies (A=0.2356, C=0.3380, G=0.2229, T=0.2035), transition/transversion ratio of 1.8958 and gamma distributed rates (shape parameter = 0.1405).

Phylogenetic trees were generated by maximum likelihood (ML) analyses, implemented in PAUP, using heuristic searches with random stepwise addition (10 replicates) and TBR (tree bisection reconnection) branch swapping. The reliability of the trees was assessed with full heuristic searches of 1000 bootstrapped datasets. Clades with bootstrap values ≥70% were considered strongly supported by the data. To determine whether the datasets from the three gene regions could be combined for phylogenetic analysis, a partition homogeneity test for congruence was done using a heuristic search in PAUP. A significant result ($P < 0.001$) indicated conflict between the data partitions and therefore a combined analysis was not possible. Therefore the three phylogenetic trees were assessed individually.

RESULTS

TRANSLATION ELONGATION FACTOR 1- ALPHA (EF1-α)

For the Tasmanian *P. ligulicola* var. *ligulicola* isolates the resulting 434 bp amplified region corresponded to base pairs 309 to 783 of *Beauveria bassiana* isolate 326 (GenBank Accession No. AY531929) and included one intron at the 5' end. The intron accounted for 204 nucleotides, of which 114 were variable, while the exon accounted for 230 nucleotides, of which 24 were variable. Most of the variability among the aligned sequences occurred in the intron. Both indels and nucleotide substitutions occurred in the intron, but only substitutions occurred in the exon. Six haplotypes were found for this region (Table 4.2). With the exception of isolate, PL4, all of the Tasmanian *P. ligulicola* var. *inoxydabilis* isolates had identical sequences for the EF1-α region (Table 4.3). Tasmanian isolate PL4 was identical to the American type culture, ATCC 10748, and the similarity between these two isolates and the remaining *P. ligulicola* isolates ranged from 68.2 to 69.0% (Table 4.3). The majority of differences between these isolates (PL4 and ATCC 10748) and the remaining *P. ligulicola* isolates occurred in the intron, as nucleotide substitutions and indels, but there

were also 12 substitutions in the exon. Sequences for both German *P. ligulicola* var. *ligulicola* isolates were identical to those of the Tasmanian *P. ligulicola* var. *inoxydabilis* isolates. The two New Zealand *P. ligulicola* var. *ligulicola* isolates were identical to each other and had 94.5% similar to the Tasmanian *P. ligulicola* var. *inoxydabilis* sequences, with 15 nucleotide substitutions in the intron and eight substitutions in the exon. The four *P. exigua* var. *exigua* isolates had 69.1 to 81.4% sequence similarity to the two varieties of *P. ligulicola* isolates (Table 4.3). Minor differences among the *P. exigua* var. *exigua* isolates occurred as one nucleotide substitution in the intron and one substitution in the exon.

INTERNAL TRANSCRIBED SPACER (ITS)

The 429 bp ITS sequences for the Tasmanian *P. ligulicola* var. *inoxydabilis* corresponded to base pairs 12 to 436 of *Ascochyta pinodes* strain MP1(GenBank Accession No. DQ383957). The sequences included ITS1, 5.8s and ITS2 regions and the combined ITS regions accounted for 271 nucleotides. The alignment revealed four haplotypes (Table 4.2). Only one difference was identified among the Tasmanian *P. ligulicola* var. *inoxydabilis* isolates, where accession AY157887 (isolate DAR 70020), originally sourced from Tasmania, differed to the remaining Tasmanian isolates by just one nucleotide substitution in ITS2. All five foreign *P. ligulicola* var. *ligulicola* isolates were identical to each other (Table 4.4). These isolates differed to *P. ligulicola* var. *inoxydabilis* by one substitution in ITS1 and similarity between the two *P. ligulicola* varieties was 99.5 to 99.8%. The *P. exigua* var. *exigua* isolates were identical to each other and similarity values between *P. ligulicola* and *P. exigua* ranged from 95.8 to 96.0%. All differences between the two species occurred in the ITS regions.

Table 4.3. Pairwise comparisons of EF1-α similarity between *Phoma exigua* (PE63210, PEMRA, PE8-25 and PE8-26) sampled from pyrethrum, *Phoma ligulicola* var. *ligulicola* (ATCC10748, DSMZ62547, DSMZ63133, ICMP2287 and ICMP10673) sampled from chrysanthemum and *Phoma ligulicola* var. *inoxydabilis* (DAR70020, PL3[b], PL4, PL12 and PL15) sampled from pyrethrum.

Isolate	PE63210	PEMRA	PE8-25	PE8-26	ATCC10748	DSMZ62547	DSMZ63133	ICMP10673	ICMP2287	DAR70020	PL3[b]	PL4	PL7	PL12	PL15
PE63210	-	99.5	99.8	99.8	69.4	81.4	81.4	80.4	80.4	81.4	81.4	69.4	81.4	81.4	81.4
PEMRA	-	-	100[a]	99.8	69.4	81.9	81.9	80.8	80.8	81.4	81.4	69.4	81.4	81.4	81.4
PE8-25	-	-	-	99.8	69.4	81.9	81.9	80.8	80.8	81.9	81.9	69.1	81.9	81.9	81.9
PE8-26	-	-	-	-	69.1	81.7	81.7	80.6	80.6	81.7	81.7	69.1	81.7	81.7	81.7
ATCC10748	-	-	-	-	-	69.0	69.0	68.2	68.2	69.0	69.0	100	69.0	69.0	69.0
DSMZ62547	-	-	-	-	-	-	100	94.5	94.50	100	100	69.0	100	100	100
DSMZ63133	-	-	-	-	-	-	-	94.5	94.50	100	100	69.0	100	100	100
NZ10673	-	-	-	-	-	-	-	-	100	94.5	94.5	68.2	94.5	94.5	94.5
NZ2287	-	-	-	-	-	-	-	-	-	94.5	94.5	68.2	94.5	94.5	94.5
DAR70020	-	-	-	-	-	-	-	-	-	-	100	69.0	100	100	100
PL3	-	-	-	-	-	-	-	-	-	-	-	69.0	100	100	100
PL4	-	-	-	-	-	-	-	-	-	-	-	-	69.00	69.0	69.0
PL7	-	-	-	-	-	-	-	-	-	-	-	-	-	100	100
PL12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	100
PL15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[a]A perfect match is indicated by 100. Decreasing values indicate less similarity.

[b] PL3 also represents *P. ligulicola* var. *inoxydabilis* isolates PL1, 46009, 50904, 52301, 58905, 58906, 62704 and 71401 sampled from pyrethrum.

Table 4.4. Pairwise comparisons of ITS similarity between *Phoma exigua* (63210, MRA, P8-25 and P8-26) sampled from pyrethrum, *Phoma ligulicola* var. *ligulicola* (ATCC10748, DSMZ62547, DSMZ63133, ICMP2287 and ICMP10673) sampled from chrysanthemum and *Phoma ligulicola* var. *inoxydabilis* (DAR70020, PL3, PL4, PL12 and PL15) sampled from pyrethrum.

Isolate	PE63210	PEMRA	PE8-25	PE8-26	ATCC10748	DSMZ62547	DSMZ63133	ICMP10673	ICMP2287	DAR70020	PL3	PL4	PL7	PL12	PL15
PE63210	-	100[a]	100	100	95.8	95.8	95.8	95.8	95.8	95.8	96.0	96.0	96.0	96.0	96.0
PEMRA	-	-	100	100	95.8	95.8	95.8	95.8	95.8	95.8	96.0	96.0	96.0	96.0	96.0
PE8-25	-	-	-	100	95.8	95.8	95.8	95.8	95.8	95.8	96.0	96.0	96.0	96.0	96.0
PE8-26	-	-	-	-	95.8	95.8	95.8	95.8	95.8	95.8	96.0	96.0	96.0	96.0	96.0
ATCC10748	-	-	-	-	-	100	100	100	100	99.5	99.8	99.8	99.8	99.8	99.8
DSMZ62547	-	-	-	-	-	-	100	100	100	99.5	99.8	99.8	99.8	99.8	99.8
DSMZ63133	-	-	-	-	-	-	-	100	100	99.5	99.8	99.8	99.8	99.8	99.8
NZ10673	-	-	-	-	-	-	-	-	100	99.5	99.8	99.8	99.8	99.8	99.8
NZ2287	-	-	-	-	-	-	-	-	-	99.5	99.8	99.8	99.8	99.8	99.8
DAR70020	-	-	-	-	-	-	-	-	-	-	99.8	99.8	99.8	99.8	99.8
PL3	-	-	-	-	-	-	-	-	-	-	-	100	100	100	100
PL4	-	-	-	-	-	-	-	-	-	-	-	-	100	100	100
PL7	-	-	-	-	-	-	-	-	-	-	-	-	-	100	100
PL12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	100
PL15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[a]A perfect match is indicated by 100. Decreasing values indicate less similarity.

Table 4.5. Pairwise comparisons of G3PD similarity between *Phoma exigua* (PE63210, PEMRA, PE8-25 and PE8-26) sampled from pyrethrum, *Phoma ligulicola* var. *ligulicola* (ATCC10748, DSMZ62547, DSMZ63133, ICMP2287 and ICMP10673) sampled from chrysanthemum and *Phoma ligulicola* var. *inoxydabilis* (DAR70020, PL3, PL4, PL12 and PL15) sampled from pyrethrum.

Isolate	PE63210	PEMRA	PE8-25	PE8-26	ATCC10748	DSMZ62547	DSMZ63133	ICMP10673	ICMP2287	DAR70020	PL3	PL4	PL7	PL12	PL15
PE63210	-	99.2	99.2	99.2	86.6	86.6	86.6	86.6	86.6	86.6	86.6	86.6	86.6	86.6	86.6
PEMRA	-	-	100[a]	100	81.7	81.7	87.1	87.1	87.1	87.1	87.1	87.1	87.1	87.1	87.1
PE8-25	-	-	-	100	81.7	81.7	87.1	87.1	87.1	87.1	87.1	87.1	87.1	87.1	87.1
PE8-26	-	-	-	-	81.7	81.7	81.7	81.7	81.7	81.7	81.7	81.7	81.7	81.7	81.7
ATCC10748	-	-	-	-	-	100	100	98.6	98.6	98.6	98.6	98.6	98.6	98.6	98.6
DSMZ62547	-	-	-	-	-	-	100	98.6	98.6	100	100	100	100	100	100
DSMZ63133	-	-	-	-	-	-	-	98.6	98.6	100	100	100	100	100	100
ICMP10673	-	-	-	-	-	-	-	-	100	98.6	98.6	98.6	98.6	98.6	98.6
ICMP2287	-	-	-	-	-	-	-	-	-	98.6	98.6	98.6	98.6	98.6	98.6
DAR70020	-	-	-	-	-	-	-	-	-	-	100	100	100	100	100
PL3	-	-	-	-	-	-	-	-	-	-	-	100	100	100	100
PL4	-	-	-	-	-	-	-	-	-	-	-	-	100	100	100
PL7	-	-	-	-	-	-	-	-	-	-	-	-	-	100	100
PL12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	100
PL15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[a]A perfect match is indicated by 100. Decreasing values indicate less similarity.

GLYCERALDEHYDE-3-PHOSPHATE-DEHYDROGENASE (G3PD)

The 363 bp region for the Tasmanian *P. ligulicola* var. *inoxydabilis* isolates corresponded to base pairs 146 to 508 of *Phoma pinodella* G3PD coding sequence (GenBank Accession No. DQ383979) and included one 82 bp intron at the 5' end. Alignment of this region identified five haplotypes (Table 4.2). Sequences for the Tasmanian *P. ligulicola* var. *inoxydabilis* isolates and the German *P. ligulicola* var. *ligulicola* isolates were identical for this region. The three remaining *P. ligulicola* var. *ligulicola* isolates from USA and New Zealand were identical to each other and differed at five nucleotide positions to the other *P. ligulicola* group, two substitutions in the coding region and three in the intron. Minor genetic variability was detected among the four *P. exigua* isolates (Table 4.5). Isolate PE63210-04 differed from the remaining three *P. exigua* isolates by one substitution and two indels in the intron region. Sequence differences between *P. exigua* and *P. ligulicola* ranged from 81.7 to 87.1 % and clearly differentiated the two species.

COMPARISON OF *PHOMA LIGULICOLA* AND *PHOMA EXIGUA* ISOLATES FROM PYRETHRUM AND CHRYSANTHEMUM WITH CLOSELY RELATED SPECIES

Sequences of EF1-α, ITS and G3PD produced in this study were aligned with sequences of closely related species from GenBank. To reduce the complexity of the analysis, only unique haplotypes (Table 4.2) were included in the comparison to related species. Due to limited availability of sequences from GenBank for the G3PD and EF1-α regions, it was not possible to compare the same species for each genomic region. Moreover, not all EF1-α haplotypes were included in the phylogenetic analyses because sequence differences among haplotypes occurred in regions that were removed to allow alignment with GenBank accessions.

Topologies produced in the three separate phylogenetic analyses placed both varieties of *P. ligulicola* and *P. exigua* relatively close to each other (Figures. 4.1, 4.2 and 4.3). *Didymella bryoniae* was placed between the two species in the ITS phylogeny and was closer to *P. ligulicola* than to *P. exigua* (Figure. 4.2). *Didymella bryoniae* was not included in the EF1-α and G3PD phylogenic analyses because no GenBank sequences were available for these regions. *Phoma ligulicola* and *P. exigua* had the greatest homology with *P. pinodella* for the inferred EF1-α phylogeny, and with *P. pinodella* and *A. pinodes* in the G3PD phylogeny (Figure. 4.3). *Phoma* species with known teleomorphs were scattered among the species whose teleomorphs were unknown.

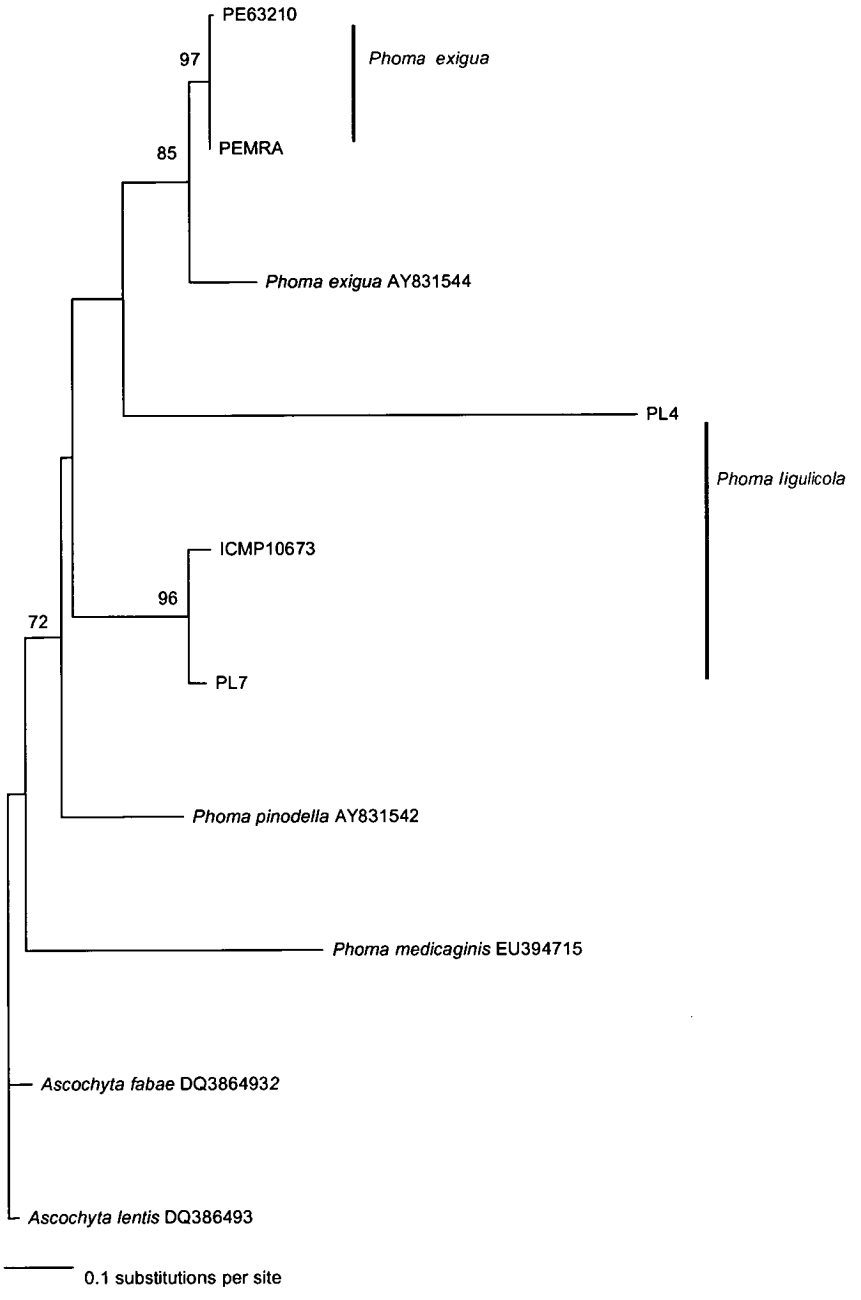

Figure 4.1. Maximum likelihood (ML) phylogeny estimated from translation elongation factor (EF1-α) sequence data for *Phoma* spp. and closely related species. Only unique haplotypes were analysed and presented here, and a complete list of isolates for each haplotype is provided (Table 4.2). Numbers at branches indicate percent bootstrap support from 1000 bootstrapped data sets (only values greater than 70% shown). Branch lengths are proportional to the inferred amount of evolutionary change. The scale bar represents 0.1 nucleotide substitutions per site.

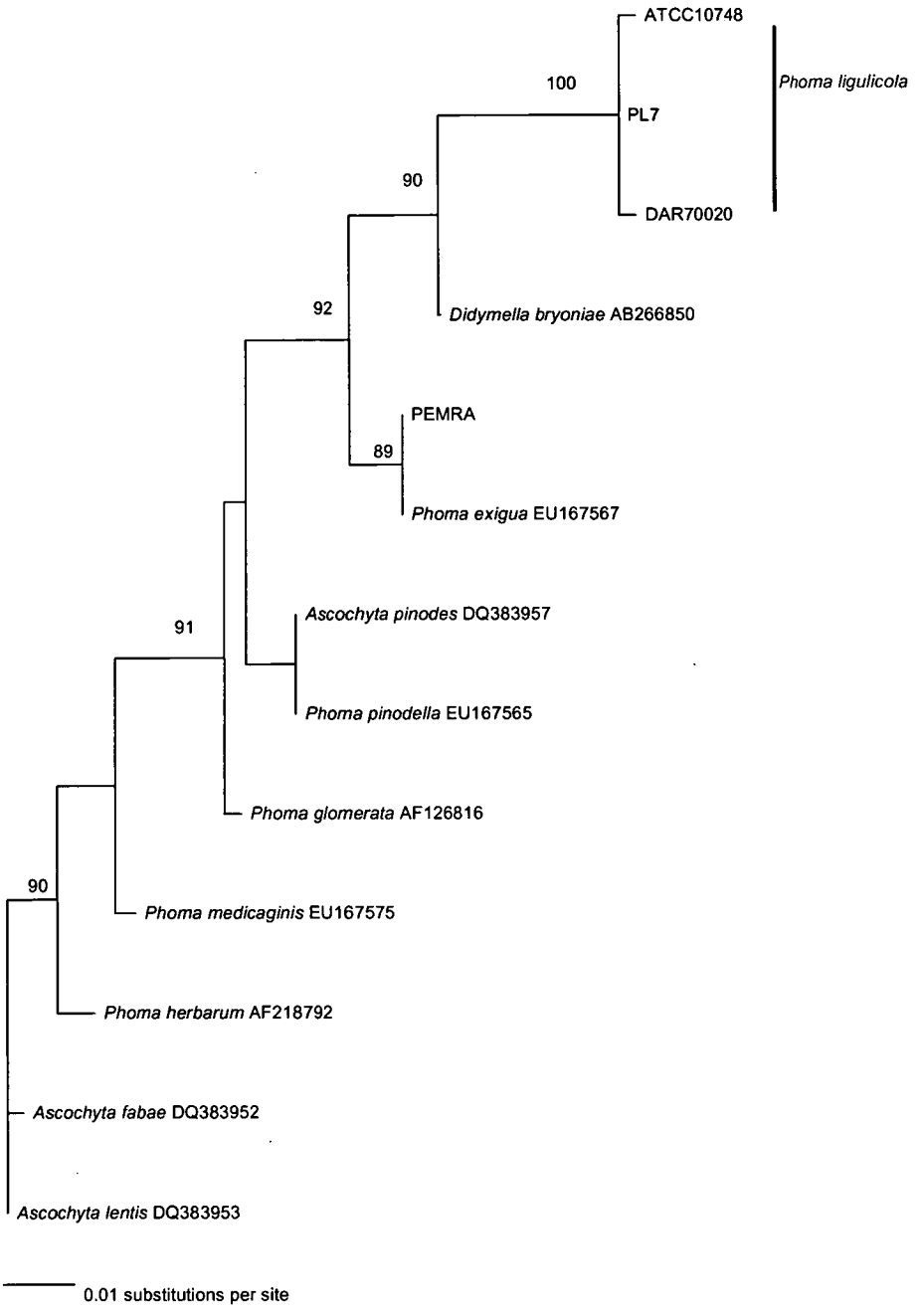

Figure 4.2. Maximum likelihood (ML) phylogeny estimated from ribosomal internal transcribed spacer (ITS) sequence data for *Phoma* spp. and closely related species. Only unique haplotypes were analysed and presented here, and a complete list of isolates for each haplotype is provided (Table 4.2). Numbers at branches indicate percent bootstrap support from 1000 bootstrapped data sets (only values greater than 70% shown). Branch lengths are proportional to the inferred amount of evolutionary change. The scale bar represents 0.01 nucleotide substitutions per site.

Figure 4.3. Maximum likelihood (ML) phylogeny estimated from glyceraldehyde-3-phosphate dehydrogenase (G3PD) sequence data for *Phoma* spp. and closely related species. Numbers at branches indicate percent bootstrap support from 1000 bootstrapped data sets. Only unique haplotypes were analysed and presented here, and a complete list of isolates for each haplotype is provided (Table 4.2). Clades were inferred from ML bootstrap values equal to or greater than 70%. Branch lengths are proportional to the inferred amount of evolutionary change. The scale bar represents 0.1 nucleotide substitutions per site.

DISCUSSION

Comparison of ITS sequences demonstrated low intraspecific variability for both species. Isolates sampled from chrysanthemum in diverse worldwide locations, and assigned to the same species and variety, had identical ITS sequences. On a smaller scale, ITS sequences from *P. ligulicola* var. *inoxydabilis* isolates sourced from various geographic regions in Tasmania also had negligible genetic variability. Accession AY157887 (isolate DAR 70020) differed by one nucleotide and no other differences were detected for the Tasmanian *P. ligulicola* var. *inoxydabilis* isolates. ITS sequences showed that the two *P. ligulicola* varieties shared greater than 99% sequence similarity. The small difference in the ITS region (one nucleotide substitution) was consistent between the two *P. ligulicola* varieties and may be useful for discrimination between them, although it will not be detectable with PCR.

The EF1-α or G3PD sequences did not clearly differentiate between *P. ligulicola* varieties, host or geographic location. The identical sequences found for the Australian and German isolates, for both the EF1-α and G3PD regions, was surprising considering the expected selection pressures associated with different hosts and geographic separation. The high level of divergence found in isolate PL4 in the EF1-α sequence was greater than expected, especially considering that this isolate was found to be identical to the remaining Tasmanian *P. ligulicola* var. *inoxydabilis* isolates in both the ITS and G3PD regions. As expected, the genetic differences found among the *Phoma* spp. isolates was greatest in the introns, but were also clearly evident in the exon region of the sequences. The intraspecific variability found in this study supports the RAPD analysis findings of Pethybridge *et al* (2005) where Tasmanian *P. ligulicola* isolate PL4 and American *P. ligulicola* isolate ATCC 10748 showed high similarity to each other and divergence from the majority of *P. ligulicola* isolates. One possible explanation for the variability may be that divergent *P. ligulicola* genotypes were introduced into Tasmania on pyrethrum

planting material sourced from various geographic locations. The phylogenies inferred for the EF1-α and G3PD sequences indicate that the *P. ligulicola* var. *ligulicola* isolates from Germany are more closely related to the Tasmanian *P. ligulicola* var. *inoxydablis* isolates than to var. *ligulicola* isolates from New Zealand. The reasons behind this are unclear, but previous intercontinental movement of chrysanthemum planting stock, means that the pathogen isolated in New Zealand is likely to have been introduced from elsewhere.

The four *P. exigua* isolates had identical ITS sequences and showed low infraspecific variability in both the G3PD and EF1-α sequences. The level of divergence in the ITS region is said to approximate speciation (Vogler and DeSalle 1994) and is generally considered to be effective for differentiating between genera and species, but not necessarily varieties or strains. Previous studies have shown that *P. exigua* varieties cannot be distinguished by sequencing of the ITS region (Abeln *et al.* 2002). The differences in the G3PD and EF1-α sequences were not consistent between isolates for the two regions and it is not yet clear whether these differences relate to biological or morphological traits.

All three regions examined in this study clearly differentiated *P. exigua* isolates from *P. ligulicola* and the individual inferred phylogenies placed the two species relatively close to each other (Figures. 4.1, 4.2 and 4.3). The partition homogeneity test indicated that the ITS, EF1-α and G3PD datasets for the *P. ligulicola* and *P. exigua* could not be combined. This was likely due to the high level of divergence in the EF1- α sequence. Compared to the topologies for ITS and G3PD, the EF1-α tree was not well supported with bootstrap confidence (Figures 4.1, 4.2 and 4.3). The bootstrap confidence for the *P. ligulicola* grouping was 100% for the ITS topology and 99% for the G3PD topology and the bootstrap confidence for the *P. ligulicola* branch of the EF1- α tree was less than 70% (Figure 4.5). However, the overall placement of *P. exigua* and *P. ligulicola* was similar for the three topologies, and the

relatedness to other *Phoma* species inferred by the phylogenetic analyses, indicates that the isolates examined in this study have been correctly identified.

For the EF1- α and G3PD sequences there was no genetic distinction between the two *P. ligulicola* varieties and separation of the *P. ligulicola* isolates for these two regions did not relate to host, or geographic separation. Hence variations within these gene regions were not suitable for differentiation of these two varieties based on the PCR primers currently available. Further work is needed to develop a molecular test for differentiating the two *P. ligulicola* varieties.

Phylogenetic analysis is a valuable supplement to traditional methods of taxonomy and systematics. To the best of my knowledge, this study presents the first EF1- α and G3PD DNA sequence data for *P. ligulicola*. Current classification of the *Phoma* genus contains little evolutionary information because it is largely based on biological and morphological data derived from only from anamorph features (Torres *et al.* 2005). The sequence information generated in this study contributes to the knowledge base of this pathogen and helps to clarify the phylogenetic affinities of *P. ligulicola* and *P. exigua* with each other and with closely related species. Additionally the regions examined in this study could potentially be used to develop specific primers which can be used for specific detection of *P. ligulicola* var. *inoxydabilis* and *P. exigua* var. *exigua* in pyrethrum crops.

CHAPTER 5

SENSITIVITY OF *PHOMA LIGULICOLA* AND *PHOMA EXIGUA* TO DIFENOCONAZOLE

INTRODUCTION

Fungicides provide the basis for control of ray light disease in Tasmanian pyrethrum crops. The current program involves the use of the sterol demethylation inhibitor fungicide difenoconazole (as Score®) and the QoI azoxystrobin (as Amistar®), in combination with a protectant, chlorathonil (as Bravo 720®). These fungicides were first introduced into pyrethrum in the year 2000. However tebuconazole (as Folicur®) has been used for control of flower diseases since 1993. Tebuconazole is in the same fungicide group as difenoconazole.

Development of resistance to particular fungicides has occurred in many pathosytems (Avenot and Michailides 2007; Erickson and Wilcox 1997; Golembiewski *et al.* 1995; Koller *et al.* 1997; Ma *et al.* 2003; Peres *et al.* 2004).
Monitoring for fungicide resistance can be used to investigate suspected cases of resistance where fungicides have failed in the field; to check that strategies to avoid development of resistance are working; to gain an understanding of the behaviour of resistant phenotypes in field populations; to monitor the spread or intensification of resistance over time or as an early warning system to assess a potential resistance problem (Brent 1988). Establishment of baseline data is required to monitor development of fungicide resistance in *P. ligulicola* var. *inoxydabilis* and for assessment of the efficacy of fungicides.

FUNGICIDE RESISTANCE

Fungicide resistance develops through one or more of the following mechanisms: (i) reduced affinity of the target site, (ii) reduced uptake or increased efflux of fungicide, (iii) detoxification, (iv) lack of conversion to active compound, (v) compensation, such as increased production of the target enzyme, and (vi) circumvention of the fungicides mode of action via an alternative metabolic pathway (Sisler 1988). Resistance can be acquired either by mutation of a single major gene or by the interaction of several mutant genes (Georgopoulos 1988). Acquisition of resistance through a single gene mutation is known as qualitative resistance. The sensitivity distribution of a pathogen population prone to this type of resistance development (disruptive selection) is discontinuous with two distinct subpopulations containing either highly sensitive or highly resistant phenotypes. Fungicide application favours the resistant strains and their numbers increase relative to the more sensitive strains (Köller and Scheinpflug 1987). By contrast, quantitative resistance development (directional selection) involves several genes and the sensitivity distributions of the initial and resulting population are continuous (Georgopoulos 1988). Qualitative fungicide resistance development can be sudden with complete loss of control, and is generally faster than quantitative responses (Köller and Scheinpflug 1987). However rates of resistance acquisition vary with selection pressure and relative fitness of the resistant phenotypes (Georgopoulos 1988).

With single gene mutation (qualitative resistance) it is difficult to detect resistant mutants in field populations until relatively high frequencies occur or are present at the time of sampling. Large, and generally impractical, numbers of samples need to be tested to give an early warning of qualitative resistance development. For example, 300 samples would need to be tested to achieve a 95% probability of detecting a 1-in-100 frequency of resistant isolates (Brent 1991). Hence without intensive sampling, resistant phenotypes are not likely to be detected unless a

large proportion of the pathogen population are resistant, and when standard fungicide applications fail to achieve satisfactory levels of disease control (Brent 1991).

With quantitative resistance, resistant strains may exist in high frequencies before loss of practical disease control is detected. An early warning of resistance development is therefore possible with smaller sample sizes (Brent 1991). Hence monitoring for DMI resistance may allow detection of resistance development before practical resistance has developed. Resistance monitoring based on one DMI is likely to be indicative of sensitivities for other DMIs because a fungus can develop cross resistance to DMIs (Köller *et al.* 1991).

In vitro bioassays are commonly used to determine fungicide sensitivity. For facultative pathogens, like *P. ligulicola*, this is done by measuring mycelial growth or spore germination on fungicide amended agar plates. A single discriminating dose of fungicide can be used, particularly for testing qualitative resistance. However testing with a range of concentrations is recommended, especially for establishment of baseline sensitivities, because it is likely to provide greater accuracy and because pathogens are exposed to a range of concentrations in the field (Brent 1991).

To quantify practical resistance, samples would ideally be collected from fields where fungicide applications had performed poorly or failed, and compared to isolates from untreated fields (Scheinpflug 1988a). However this sampling procedure is difficult to adopt for the pyrethrum industry because all fields are treated routinely to prevent serious epidemics and disease management strategies have been largely successful (Pethybridge *et al.* 2008e). Consequently, fungicide sensitivity data derived from isolates from fields where fungicide applications have performed adequately represents baseline sensitivity distributions and does not

translate into definitive levels of resistance or fungicide dosage recommendations. Establishment of baseline sensitivity data enables monitoring of shifts in fungicide sensitivity of a pathogen population. Collection of one or a few isolates per field from a considerable number of crops is generally sufficient for initial resistance monitoring (Brent 1991).

This chapter examined fungicide sensitivity of *P. ligulicola* var. *inoxydabilis* and *P. exigua* var. *exigua* isolates to difenoconazole. Two scales of *Pho ma* spp. populations were assessed, a within-field and a between-field population. The between-field population of *P. ligulicola* var. *inoxydabilis* isolates were single samples collected from different fields and at different times. As such these isolates are a regional fungal population of geographically and temporally separated individuals. These fungal isolates represent a small portion of the genotypes present at a given time and may or may not contain highly sensitive or resistant subpopulations. Potentially there could be numerous genetically and biologically diverse *P. ligulicola* individuals within one field, on one plant, leaf or stem at any given time. The within-field population provided a comparable assessment of fungicide sensitivity on a relatively small spatial and temporal scale.

The main objective of this study was to:

1. Determine the baseline sensitivity of *P. ligulicola* var. *inoxydabilis* and *P. exigua* isolates, collected from pyrethrum crops, to difenoconazole.
2. Determine the distributions of sensitivities among two *P. ligulicola* var. *inoxydabilis,* a between-field and a within-field population.

A secondary objective was to determine the sensitivity of *P. ligulicola* var. *ligulicola* isolates sourced from chrysanthemum plants which have had little or no exposure to DMIs and thus provide additional baseline information.

METHODS AND MATERIALS

ISOLATES USED IN THIS STUDY

Isolates from pyrethrum

The between-field population (53 isolates) was isolated from diseased stems, leaves and buds collected from 52 pyrethrum fields across northern Tasmania during 2003 and 2004 and an additional isolate, DAR70020 was collected in 1995. The within-field population (58 isolates) was collected from pyrethrum showing symptoms of ray blight disease in three untreated plots in a fungicide trial at Wesley Vale during October and December 2006. These two populations were likely to have been exposed to DMI fungicides because tebuconazole has been routinely used on Tasmanian pyrethrum crops since 1993 and difenoconazole since 2000.

Four *P. exigua* var. *exigua* isolates were also tested for sensitivity to difenoconazole. Two isolates were collected from different fields in 2004 and the other two were collected from the same field as the within-field *P. ligulicola* var. *inoxydabilis* isolates in 2006. These isolates could also have been exposed to DMIs.

Isolates from chrysanthemum

Isolates were sourced from herbariums in the American Type Culture Collection, Manassas, VA, USA (ATCC10748), the Deutche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany (DSMZ62547 and DSMZ63133) and Landcare Research, Auckland, New Zealand (ICMP2287 and ICMP10673). Cultures from the USA, Germany and New Zealand were isolated from ornamental chrysanthemum. The cultures from the USA and Germany were collected prior to the introduction of DMI fungicides in the early 70s and would not have been exposed to DMIs. The New Zealand cultures were isolated in 1978 and may have been exposed to DMIs.

DIFENOCONAZOLE SENSITIVITY *IN VITRO* ASSAY

Sensitivity of each isolate to difenoconazole was assessed by the inhibition of mycelial growth. A stock solution of difenoconazole (50,000 µg a.i./ml) was made by dissolving 2 ml of Score 250® (Syngenta Australia) in 2 ml of sterile distilled water mixed with 6 ml of ethanol. Further stock solutions (5000, 500, and 50 µg a.i./ml) were made by serial dilutions in sterile distilled water. Aliquots were added to autoclaved PDA (precooled to 55°C) to give final concentrations of 0, 0.1, 0.4, 0.16, 2.5, 50 and 100 µg/mL. Amended plates were inoculated with 3-mm-diameter plugs of mycelium growing on PDA. Cultures were refreshed 10 to 14 days prior to inoculation. Three plates of each concentration were tested for each isolate. Inoculated plates were incubated in darkness at 20°C and mycelial growth was assessed three times per week. Colony diameters were measured when the control plates were close to the edge of the petri dish (>78 mm) or after 5 weeks for slow growing cultures. Growth rates were expressed as mm per day.

DATA ANALYSIS

The sensitivity of each isolate was determined by probit analysis, which calculated the effective concentration of active ingredient required to cause 50% inhibition (EC_{50}) of mycelial growth rate. Inhibition was calculated as (=1- (mean growth rate on amended media divided by the mean growth rate on unamended media)). Probit analyses were conducted using a generalised form of the Macro written by (Hsiang *et al.* 1997) for the Statistical Analysis System (Version 9.0). The values used for Probit analysis were the mean of three replicates of each concentration. The Mann-Whitney *U*-test was used to compare the medians of the EC_{50} values for the between-field and within-field *P. liguicola* var. *inoxydabili*s populations.

RESULTS

The EC_{50} distributions of the between-field *P. ligulicola* var. *inoxydabilis* population (n = 53) were significantly higher than those of the within-field (n = 58) isolates (P < 0.001). Frequency distributions for these two populations are presented in Figure 5.1. The EC_{50} values for the between-field population ranged from 0.047 to 0.446 µg/ml, with a mean EC_{50} value of 0.167 µg/ml. For the within-field population, EC_{50} values ranged from 0.059 to 0.200 with a mean EC_{50} value of 0.108 µg/ml. There was no significant difference between the median EC_{50} values of the two *P. ligulicola* var. *inoxydabilis* populations (z = -1.130; P=0.259).

The five *P. ligulicola* var. *ligulicola* isolates from chrysanthemum had EC_{50} values from 0.039 to 0.119 µg/ml (Table 5.1). The two isolates from New Zealand, which may have been exposed to DMIs, had lower EC values than the unexposed isolates from Germany. Forty-six percent of the Tasmanian *P. ligulicola* population was within the EC_{50} range of the three *P. ligulicola* from chrysanthemum and without a history of DMI exposure.

The EC_{50} values of the four *P. exigua* isolates ranged from 0.802 to14.526 µg/ml (Table 5.1). The EC_{50} value of *P. exigua* isolate PE8-25 was 32 and 72 times greater than for the highest EC_{50} values for the between-field and within-field populations *P. ligulicola* populations respectively. The EC value for the most sensitive *P. exigua* isolate (PEMRA) was almost twice that of the least sensitive *P. exigua* isolate.

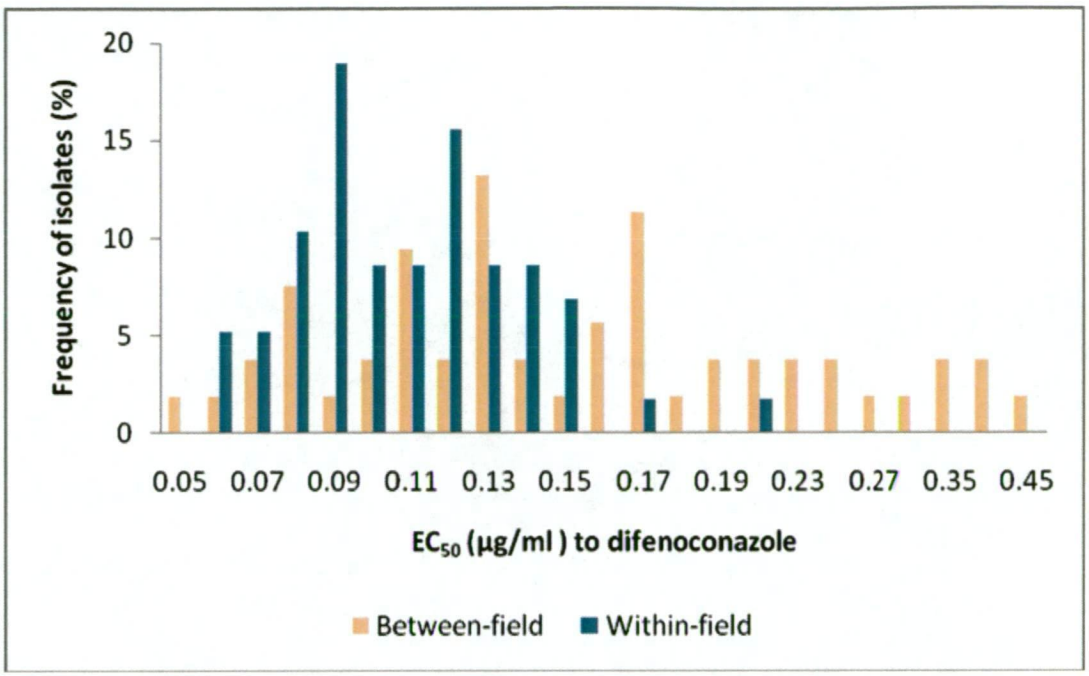

Figure 5.1. Frequencies distributions of effective concentrations of difenoconazole to inhibit 50% of mycelial growth (EC_{50}) values for 53 *Phoma ligulicola* within-field isolates collected from 52 pyrethrum crops in Tasmania and for 58 *Phoma ligulicola* within-field isolates from one pyrethrum crop in Tasmania.

Table 5.1. EC_{50} values of five *Phoma ligulicola* var. *ligulicola* isolates from chrysanthemum and four *Phoma exigua* var. *exigua* isolates from pyrethrum in Tasmania.

Isolate	Date of collection	Location	EC_{50} (µg/ml)
Phoma ligulicola var. *ligulicola* from chrysanthemum			
ATCC 10748	Early 1970s	USA	0.083
DSMZ 62547	Early 1970s	Germany	0.119
DSMZ 63133	Early 1970s	Germany	0.100
ICMP 2287	1978	New Zealand	0.039
ICMP 10673	1978	New Zealand	0.091
Phoma exigua var. *exigua* from pyrethrum			
PEMRA	September 2004	Sassafrass	0.802
PE63210	September 2004	Wesley Vale	10.524
PE8-25	October 2006	Wesley Vale	14.526
PE8-26	October 2006	Wesley Vale	3.882

DISCUSSION

The current disease management approach of the pyrethrum industry relies heavily on the use of fungicides because there are no sources of resistant plant material and cultural disease control methods used by the chrysanthemum industry are not practical for the pyrethrum industry. This is the first study to characterise baseline sensitivity to difenoconazole in *P. ligulicola* var. *inoxydabilis* populations obtained from pyrethrum in Australia. This is valuable information required for establishing a programme to effectively monitor fungicide resistance development and hence assess the impact of fungicide use.

This study found that the Tasmanian *P. ligulicola* var. *inoxydabilis* population was generally sensitive to difenoconazole. The sensitivity distributions were continuous for both the within-field and the between-field *P. ligulicol a* var. *inoxydabilis* populations from pyrethrum (Figure 5.1). Resistance development to DMIs is a gradual directional selection towards a less sensitive population (Scheinpflug 1988b; Smith *et al.* 1991). Selection of DMI resistant populations appears to involve factors other than the mode of action, such as the type of selection of resistant subpopulations (Köller and Sch einpflug 1987). The frequency distribution of sensitivities to DMI fungicides in unexposed pathogen populations is continuous with phenotypes that range from highly sensitive to considerably less sensitive than the population mean (Köller and Scheinpflug 1987).

The between-field population had a greater range of sensitivities and generally higher EC_{50} values than the within-field population. Some of the variability among the between-field isolates would be due to natural variation and phenotypic responses. Variation in the degree of DMI exposure is also likely to have contributed to the greater range of sensitivities in the between-field population. Selection pressure for reduced fungicide sensitivity is high for fungal populations that are exposed to particular fungicides over longer periods of time (Brent 1988).

Pyrethrum is a perennial crop, with a lifetime of three to four years, so isolates from the between-field population may have been exposed to 16 applications of DMI fungicides. In comparison, the within-field population was collected from a field approaching first year harvest. Isolates collected from this crop would have been exposed to a maximum of four DMI applications in 2005, two applications of difenoconazole in spring and two applications of tebuconazole in summer. Hence the between-field population was likely to have isolates with higher EC_{50} values than the within field population.

Quantification of resistance requires comparison of isolates with no exposure to a particular fungicide with those from fields where this fungicide has failed to control the disease (Koller *et al.* 1997). Exposure to a given fungicide provides selection pressure that favours survival and reproduction of the less sensitive isolates of the population. This causes a 'shift' in the distribution of sensitivities towards a more resistant population (Köller and Scheinpflug 1987). The efficacy of the fungicide decreases as the fungal population becomes more resistant. Practical resistance occurs when the proportion of resistant phenotypes in a population increase to levels where standard fungicide applications fail to achieve commercially acceptable disease control (Köller *et al.* 1991). It is not possible to classify the *P. ligulicola* var. *inoxydabilis* isolates as 'sensitive' or 'resistant' because no isolates have been collected from pyrethrum crops showing evidence of practical resistance.

However, based on other studies of fungicide sensitivity, the EC_{50} values for these two *P. ligulicola* populations were comparatively low. For example *Alternaria alternata* isolates from pistachio orchards had EC_{50} values >500 µg/ml for the carboxamide fungicide, boscalid (Avenot *et al.* 2008). Control failures were not reported for these orchards, but boscalid had been applied (in a mixture with the strobilurin, pyraclostrobin) two or three times per season for up to four years.

The five *P. ligulicola* var. *ligulicola* isolates from chrysanthemum were also sensitive to difenoconazole. Despite the small sample size of isolates investigated, and the relatively low EC_{50} values, this study found variation in fungicide sensitivity among three isolates with no previous history of exposure to difenoconazole. The low EC_{50} values for the isolates from New Zealand suggest that these isolates have had little or no exposure to DMI fungicides. Fifty-four percent of the Tasmanian *P. ligulicola* var. *inoxydabilis* population had higher EC_{50} values than the three *P. ligulicola* var. *ligulicola* isolates from chrysanthemum with no prior history of DMI exposure. This indicated that there was a slight shift towards reduced sensitivity to difenoconazole for the Tasmanian *P. ligulicola* var. *inoxydabilis* population. This shift in sensitivity is likely due to prolonged use of DMI fungicides, such as tebuconazole, for over 10 years. The definition of practical resistance is met when the number of phenotypes with reduced sensitivity increase to a level that results in unsatisfactory fungicide control in the field (Köller, 1991). None of the isolates in this study originated from fields in which disease management practices had failed. However, the inclusion of other fungicides such as azoxystrobin in the disease management recommendations may make the failure of DMIs such as difenoconazole to control ray blight disease hard to detect. Determination of baseline sensitivities for individual fungicides is therefore an important initial step in monitoring changes in sensitivity over time and will assist in development of long-term disease management strategies.

Even with the limited sampling of this study, the reduced sensitivity to difenoconazole found in the *P. exigua* var. *exigua* isolates is of potential concern. The *P. exigua* var. *exigua* isolates had high EC_{50} values compared to *P. ligulicola* isolates (Figure 5.1; Table 5.1) and none of the four *P. exigua* isolates from pyrethrum were highly sensitive to difenoconazole. The *P. exigua* population may naturally have a wider range of sensitivities than *P. ligulicola*. Alternatively, *P. exigua* populations may have had previous long term exposure to DMIs. This

ubiquitous pathogen may be infecting hosts other than pyrethrum, including commercial vegetable crops treated with difenoconazole and other DMIs. For example, *Phoma exigua* var. *exigua* causes gangrene of potatoes (Boerema *et al.* 2004) and is found on potato crops in Tasmania (Walker and Wade 1976). Difenoconazole (Score®) is applied to potatoes in Tasmanian to control Target Spot (Early Blight) caused by *Alternaria solani* and several applications may be applied in a season (Simon Jones *pers. comm.*).

The isolation frequency of *Phoma exigua* var. *exigua* has been much lower than for *P. ligulicola* var. *inoxydabilis.* Hence the incidence of *P. exigua* may not be high enough to cause a serious level of disease or loss of fungicide effectiveness. However this may change for example if inoculum builds up in fields or in years of particularly conducive environmental conditions. Furthermore, the frequency of this species on pyrethrum could increase through selection pressure on phenotypes already showing reduced sensitivity to difenoconazole. Acquisition of fungicide resistance often comes at the cost of reduced pathogen 'fitness' in terms of mycelial growth, reproduction and pathogenicity (Köller 1988). Hence fungicide resistant strains or genotypes may be less able to survive and cause disease than the sensitive strains. It is important to determine if this is the case for the *P. exigua* var. *exigua* isolates found on pyrethrum.

CHAPTER 6

VARIATION IN PATHOGENICITY OF *PHOMA* SPECIES FROM PYRETHRUM AND CHRYSANTHEMUM

INTRODUCTION

Breeding disease resistant pyrethrum cultivars has the potential to provide the Tasmanian pyrethrum industry with a sustainable and economical alternative to fungicide control of ray blight disease, caused by *P. ligulicola* var. *inoxydabilis*. Successful resistance breeding programs require an understanding of interactions between the host and the pathogen population. Characterisation of pathogenic variability within a pathogen population enables identification of virulent isolates which can then be used to screen potential plant material for susceptibility or resistance.

The pyrethrum breeding program in Tasmanian is mainly concerned with breeding for traits that influence pyrethrins content of flowers, flower yield and the time it takes for flowers to reach harvest maturity. However the breeding program also aims to identify cultivars with reduced susceptibility to ray blight (K. Groom *pers. comm.*). Differences in susceptibility of commercial cultivars and in parent lines to ray blight were detected in field trials during the 2006 and 2007 pyrethrum seasons (Pethybridge *et al.* 2008d). Reliable and cost effective methods of evaluating potential cultivars are needed to supplement and validate field based data, and to guide selection of disease resistant cultivars for future field trials and eventual commercial use.

Previous studies of *P. ligulicola* var. *inoxydabilis* on pyrethrum have focused on various epidemiological aspects of natural disease development in the field including spatial and temporal patterns of disease epidemics (Pethybridge *et al.* 2005a), the influence of site factors on disease incidence (Pethybridge and Hay 2001) and seasonal fluctuations in disease severity and frequency (Pethybridge *et al.* 2003). Pyrethrum cultivar susceptibility to ray blight disease has been examined in the field over several seasons and in a glasshouse study (Pethybridge *et al.* 2007b). While no resistant commercial cultivars have been identified, cultivars have been found to vary in their susceptibility to ray blight in the field (Pethybridge *et al.* 2008f; Pethybridge *et al.* 2007b). However, little is known about the variability in virulence of the Tasmanian *P. ligulicola* var. *inoxydabilis* population. Additionally, previous plant inoculation based studies of this pathogen have used one or few isolates whose pathogenicity had not been characterised.

Inoculation of greenhouse plants with conidial suspensions is a widely used technique for assessing pathogenic traits and host susceptibility (Allingham and Jackson 1981; Benedikz *et al.* 1981; Chen *et al.* 2005; Kaiser *et al.* 1997; Pethybridge *et al.* 2004a). However this method is limited to fungal isolates which produce sufficient quantities of spores. Additionally there are quarantine issues that need to be considered when handling pathogen species and strains that have been sourced from regions outside the testing area and the risk of introducing new species. *In vitro* methods of inoculating detached plant parts, such as leaves or stems, enable testing of foreign isolates in a contained environment. Inoculation of detached plant material with conidial suspensions is one such method, however it is also limited to isolates that sporulate reliably. Inoculation of intact plants or detached plant material with hyphae, with or without physical wounding of the plant, is an alternative method that has been successfully used for *P. ligulicola* (Chesters and Blakeman 1967; McCoy 1971; Pethybridge and Wilson 1998). To enable testing of isolates that do not sporulate, a detached leaf assay, which

involved inoculation of leaves with colonised toothpicks, was adapted from the method used by McCoy (1971).

This chapter examined the pathogenic variability of *Phoma* isolates from Tasmanian pyrethrum crops in terms of lesion growth on detached leaves and disease incidence on inoculated potted plants.

The aims of this study were to:

1. Characterise pathogenic variability of Tasmanian *P. ligulicola* var. *inoxydabilis* isolates obtained from diseased commercial pyrethrum.
2. Confirm the pathogenicity of *P. exigua* var. *exigua* and *P. ligulicola* var. *ligulicola* isolates to commercial pyrethrum.
3. Determine if there are correlations between virulence data obtained for the detached leaf assay and the greenhouse disease incidence trial.

METHODS AND MATERIALS

ISOLATES USED IN THIS STUDY

Twenty three *P. ligulicola* var. *inoxydabilis* isolates and two *P. exigua* var. *exigua* isolates obtained from *T. cinerariifolium* in Tasmania (outlined in Chapter 3) were chosen for virulence testing. To provide a range of phenotypes, isolates were selected to encompass a range of morphological variability and arising from geographically separated pyrethrum crops. Five *P. ligulicola* var. *ligulicola* isolates obtained from chrysanthemum ray blight and sourced from various international culture collections were included in the detached leaf assay. The foreign *P. ligulicola* var. *ligulicola* isolates were confined to the *in vitro* virulence assay and were not be used in the greenhouse experiment due to the risk of inadvertently introducing this pathogen into the Tasmanian pyrethrum. Sixteen *P. ligulicola* var. *inoxydabilis* isolates and the two *P. exigua* isolates, from the *in vitro* detached leaf assay, were screened for aggressiveness in the greenhouse study.

The *P. exigua* var. *exigua* isolates used in this study were isolated from commercial pyrethrum stem lesions which were typical of infection with ray blight. At the time it was not clear whether infection with *P. exigua* var. *exigua* had caused the lesion or if this fungus was colonising necrotic tissue already damaged by *P. ligulicola* var. *inoxydabilis.* It is possible that both fungi were present at the time of isolation. Two *Phoma exigua* var. *exigua* isolates were included in this study to confirm the pathogenicity of this fungus to pyrethrum and to provide a comparative *Phoma* species. Seven *P. ligulicola* var. *inoxydabilis* isolates (from the 23 isolates used in the detached leaf assay) could not be screened in the greenhouse study because they produced little or no conidia. Hence sufficient quantities of inoculum, at the required concentration, could not be made for these seven isolates.

DETACHED LEAF ASSAY

Leaves were collected from six month old pyrethrum (cultivar 'A') plants on the day of inoculation. The plants were grown from seed and maintained in pots in a greenhouse at 15 to 20°C under a sprinkler irrigation system. The leaves were rinsed in two changes of dH_2O and then soaked in methylated spirits for three to four minutes and then air-dried in a laminar flow for 10 minutes before being inoculated with toothpicks with fungus growing on them. Autoclaved toothpicks were inoculated by placing the tips into fungal cultures which were growing on potato dextrose agar and incubating them in darkness for 16 hours at 21°C. Fungal cultures were refreshed on potato dextrose agar five to seven days before inoculating toothpicks. The inoculated toothpicks were forced into the leaves near a leaf axil and left for the duration of the incubation period. Inoculated leaves were placed into 50 ml screw-top centrifuge tubes which contained 15 ml of 2% water agar. Leaves pierced with non-inoculated toothpicks, and leaves with no toothpick were used as controls. Each leaf was placed into a separate tube with the leaf pushed 1 to 2 cm down into the agar to keep it upright and away from the sides of

the tube. Lids were secured onto the tubes to maintain humidity and prevent contamination during the incubation period and incubated in darkness at 21°C. The tubes were placed in racks to keep them upright. The experiment was a completely randomised design. Four leaves were inoculated for each of the 30 isolates and for both controls. The experiment was done twice. After five days the leaves were removed from the tubes and lesions were measured. Because the leaves were narrow (1 to 2 mm diameter) only lesion length was measured. Lesion length was classified as the length of necrotic tissue that extended out from either side of the inoculation site (Figure 6.1). Isolations were conducted from one diseased leaf for each isolate to verify that the lesions were cause by the *Phoma* isolates. Small sections (less than 2 mm) of leaf were excised from a junction of diseased and healthy tissue and placed onto 2% water agar. After 48 h incubation at 21°C in darkness, single hyphal tips were isolated from the resulting fungal growths and transferred to potato dextrose agar for cultural examination.

Figure 6.1. Lesions and aerial mycelia on toothpick inoculated detached pyrethrum leaves 5 days after inoculation with *Phoma ligulicola* var. *inoxydablis* isolate 52301.

GREENHOUSE ASSAY

The pathogenicity of 16 *P. ligulicola* var. *inoxydabilis* isolates and two *P. exigua* var. *exigua* isolates, all obtained from pyrethrum, were tested on the same commercial pyrethrum cultivar that was used for the detached leaf assay. The plants were grown from the same seedlot as the detached leaf assay and maintained in the greenhouse under the same conditions. The inoculum was a conidial spore suspension. Conidia were collected from cultures grown on V8 agar at 20°C under fluorescent lighting for 10 to 14 days. Three to four small blocks (~10 mm^3) of V8 agar containing pycnidia were placed into autoclaved McCartney bottles containing 5 ml of autoclaved dH_2O. Conidia were released from pycnidia by gently agitating the bottles by hand. The number of spores were estimated with a haemocytometer and spore concentrations were adjusted to 5 x 10^5 conidia per ml water. The wetting agent, Tween 20, was added to the inoculum at a rate of 1 drop per 100 ml water. The foliage of 8 week old plants was sprayed with approximately 3 ml of inoculum, using a hand held spray bottle. Control plants were sprayed with the same volume of water and Tween 20 as the inoculated plants. Plants were covered with plastic bags immediately after spraying to maintain high humidity and conditions conducive for infection for 48 h. There were five replicated pots, each with four plants, for each isolate. An additional five pots with four plants were included as control plants. A completely randomised design was used to distribute the pots on the greenhouse bench. To reduce the risk of cross contamination, pots were spaced so that foliage from one plant did not touch another. All isolates were tested three times during a four month period in 2007. The first trial was done as two split plots with 10 isolates screened in the first week of February and the remaining eight isolates screened in the third week of February. The second and third trials were conducted in April and May with all 18 isolates screened simultaneously in both trials. To minimise variability in environmental conditions, all trials were carried out on the same bench which remained in the same position in the greenhouse for the duration of the experiment.

The viability of conidia used in the inoculum was assessed by pipetting 200 µl of inoculum onto 2% water agar in petri plates. Spores were spread over the surface of the agar using a sterile glass rod and plates were incubated in darkness at 21°C for 24 h. For each isolate, 100 conidia within at least 10 randomly selected fields of view were assessed for germination under ×200 magnification. Spores were considered viable if the germ tube was at least half as long as the conidium it emerged from.

Disease incidence was assessed 15 days after inoculation by recording the proportion of leaves showing ray blight symptoms (Figures 6.2 and 6.3) or lesions caused by *Phoma exigua* . The number of diseased leaves and the total number of leaves on each plant were counted, and the percentage of diseased leaves (disease incidence) was calculated for each plant. Isolations were conducted (as described above) from four leaves (of inoculated plants showing typical ray blight symptoms) for each isolate over the three trials. Isolations were also done on four leaves of control plants when lesions were found. To ascertain that *Phoma* spp. symptoms were correctly identified, additional isolations were done on any plants with lesions not characteristic of the two *Phoma* species. For example light tan or rust coloured lesions or yellowing of stems.

MYCELIAL GROWTH ON PDA

As an additional component of this assay, the growth of all 30 isolates on potato dextrose agar was measured because mycelial growth rate on PDA and lesion growth on chrysanthemum stems were found to be correlated (McCoy 1971). For each isolate 3 petri dishes containing potato dextrose agar were inoculated with 5 mm plugs taken from the same agar plates that were used to inoculate the toothpicks. Inoculated plates were incubated in darkness at 2°C. Colony diameters were measured after 7 days incubation and mycelial growth (mm/day) was

calculated as the diameter, minus the inoculation plug, divided by the number of days.

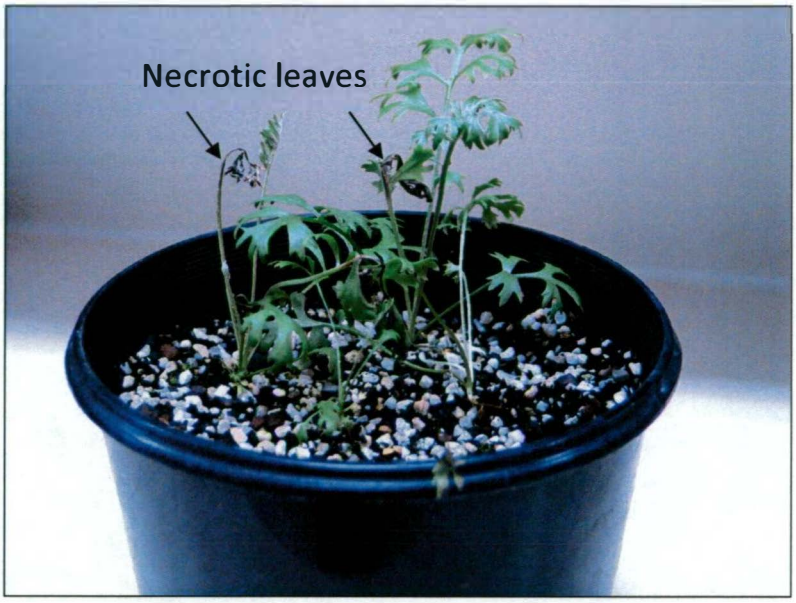

Figure 6.2. Necrotic leaves of pyrethrum seedlings inoculated with conidial inoculum from *Phoma ligulicola* var. *inoxydabilis* isolate 62704.

Figure 6.3. Necrotic leaf and characteristic pinched stem of seedling inoculated with conidial inoculum from *Phoma ligulicola* var. *inoxydabilis* isolate 62704.

EXPERIMENTAL DESIGN AND DATA ANALYSIS

All glasshouse and growth chamber experiments were conducted using completely randomized designs. For the detached leaf assay, lesion growth was calculated as the lesion length divided by five to give mm/day and each leaf represented one experimental unit. Analysis of variance was implemented for all data sets using the program SAS version 9.1 (SAS Institute Inc., Cary, NC, USA.). Mycelial growth rates were analysed using one way ANOVA. Tukey's test at 0.05 significance was used to compare isolate means for the detached leaf assay and mycelial growth. For the greenhouse experiment, disease incidence was calculated for each plant and the scores of four plants in one pot were averaged to represent one experimental unit. To account for the unbalanced nature of this data set (the first of the three trials was split) the data were analysed using a mixed general linear model in SAS and the simulation method was used to separate isolate means at 0.05 and 0.10 significance. Residuals were plotted against observed values and predicted values to check for departure from normality for the three data sets. Pearson's correlation coefficient was used for correlation between disease intensity, lesion growth and mycelial growth and the average scores of each isolate, for each experiment were used.

RESULTS

DETACHED LEAF ASSAY

After five days inoculated leaves showed lesions (dark necrotic plant tissue) which were clearly visible as a distinct darkening of an otherwise green leaf (Figure 6.1). White aerial mycelia were visible on many lesions, with some isolates consistently producing more mycelia than others. Leaves that had not been pierced with a toothpick were free of lesions and aerial mycelium. On leaves with non-colonised toothpicks, darkened tissue was visible approximately 1 mm either side of the

puncture wound and no aerial mycelia were detected. These lesions were likely due to a wound response and not associated with *Phoma*. All leaves showed some yellowish-brown discolouration on the area that was inserted into water agar and this was also likely due to a wound response (Figure 6.1). All isolations from diseased tissue confirmed that the lesions were caused by the intended fungus and the re-isolations were visually comparable to the original cultures which were generally distinct in appearance. The 30 *Phoma* isolates showed significant ($P < 0.001$) variation in virulence on pyrethrum cultivar 'A' (Table 6.1). Mean lesion growth of the 23 Tasmanian *P. ligulicola* var. *inoxydablis* from pyrethrum ranged from 3.0 to 6.7 mm/day. Mean lesion growth of the five *P. ligulicola* var. *ligulicola* isolates from chrysanthemum varied from 4.6 to 6.4 mm/day and means for these isolates were indiscriminately distributed among the Tasmanian *P. ligulicola* var. *inoxydabilis* isolate means. Compared to the *P. ligulicola* isolates, the *Phoma exigua* isolates from pyrethrum showed relatively slow lesion growth of 2.6 and 4.6 mm/day.

GREENHOUSE ASSAY

Analysis of variance showed a significant ($P < 0.001$) difference in disease incidence among isolates, but all pairwise comparisons (separation) of least squares means did not reveal any significant differences between isolates at alpha 0.05. Significant differences in disease incidence means were however evident at alpha 0.1 (Table 6.1). Disease incidence for 16 *P. ligulicola* var. *inoxydabilis* and two *P. exigua* var. *exigua* isolates ranged from 8.2 to 19.7 % and from 9.5 to 10.7 %

respectively. Disease incidence was continuous among the isolates with no obvious aggregation of high or low values. On non-inoculated control plants 2.0 % of leaves had necrotic spots not characteristic of *P. ligulicola* var. *inoxydabilis* or *P. exigua* var. *exigua*. Several plants, including the non-inoculated control plants, had tan coloured necrotic leaf tips or leaf lesions. Isolations from these symptoms

Table 6.1 Mean lesion growth for the detached leaf assay (lesion growth) and disease incidence (percentage of diseased leaves) for the greenhouse experiment.

Isolate code	Host plant	Location	Lesion growth[1]	Disease incidence[2]	Mycelial growth[3]
Phoma ligulicola var. *inoxydabilis*					
46009	*T. cinerariifolium*	Forth, Tas.	6.05 abc	-	8.07 abcde
50001	*T. cinerariifolium*	Kindred, Tas.	6.19 abc	19.66 ab	7.19 fghi
52301	*T. cinerariifolium*	Penguin, Tas.	5.67 abc	16.80 ab	7.02 ghi
55504	*T. cinerariifolium*	Unknown, Tas.	6.70 a	19.70 a	7.67 defgh
57312-04	*T. cinerariifolium*	Sassafrass, Tas.	4.48 bcdef	8.45 b	7.29 efghi
58905	*T. cinerariifolium*	Unknown, Tas.	4.18 cdef	8.28 b	4.50 mn
58905-SD2	*T. cinerariifolium*	Wesley Vale, Tas.	5.48 abc	-	7.86 bcdef
58906-04	*T. cinerariifolium*	Wesley Vale, Tas.	5.36 abcd	17.47 ab	6.05 jk
59302	*T. cinerariifolium*	Wesley Vale, Tas.	4.41 bcdef	-	7.83 bcdef
62606-04	*T. cinerariifolium*	Wesley Vale, Tas.	4.31 cdef	12.36 ab	4.55 mn
62704	*T. cinerariifolium*	Sassafrass, Tas.	5.24 abcd	17.05 ab	4.81 1m
62704B	*T. cinerariifolium*	Sassafrass, Tas.	5.89 abc	19.18 ab	6.07 jk
66601B	*T. cinerariifolium*	Sassafrass, Tas.	5.20 abcd	15.68 ab	5.79 kl
68501	*T. cinerariifolium*	Wynyard, Tas.	5.98 abc	18.23 ab	7.19 fghi
71401	*T. cinerariifolium*	Kindred, Tas.	3.36 def	-	8.19 abcd
DAR70020	*T. cinerariifolium*	Forth, Tas.	4.95 abcde	12.10 ab	7.69 defgh
PL1	*T. cinerariifolium*	Scottsdale, Tas	4.85 abcde	15.48 ab	4.52 mn
PL3	*T. cinerariifolium*	Cressy, Tas	2.99 ef	-	6.64 ij
PL4	*T. cinerariifolium*	Deloraine, Tas	5.05 abcde	-	7.00 hi
PL7	*T. cinerariifolium*	Burnie, Tas.	4.71 abcdef	15.43 ab	5.24 lm
PL12	*T. cinerariifolium*	Wynyard, Tas.	5.99 abc	15.21 ab	7.48 defgh
PL13	*T. cinerariifolium*	Sassafrass, Tas.	5.10 abcde	8.81 b	7.81 cdefg
PL15	*T. cinerariifolium*	Wynyard, Tas.	5.76 abc	-	7.21 fghi

Isolate code	Host plant	Location	Lesion growth[1]	Disease incidence[2]	Mycelial growth[3]
Phoma exigua var. *exigua*					
PE63210-04	*T. cinerariifolium*	Wesley Vale, Tas.	2.63 f	10.76 ab	3.91 n
PEMRA	*T. cinerariifolium*	Wesley Vale, Tas.	4.63 abcdef	9.49 ab	3.00 o
Phoma ligulicola var. *ligulicola*					
ATCC 10748	*Dendrathema* × *grandiflorum*	North Carolina, USA	4.62 abcdef	-	8.71 a
DSMZ62547	*D.* × *grandiflorum*	Germany	6.42 ab	-	8.79 a
DSMZ63133	*D.* × *indicum*	Germany	5.49 abc	-	8.55 abc
ICMP10673	*D.* × *grandiflorum*	New Zealand	6.24 abc	-	8.14 abcd
ICMP2287	*D.* × *grandiflorum*	New Zealand	4.70 abcdef	-	8.62 ab

1. Each value represents the mean of eight detached leaves per isolate. Means followed by the same letter/s are not significantly different at $P = 0.05$ according to Tukeys's HSD test.
2. Each value represents the mean of 60 plants per isolate. Means followed by the same letter/s are not significantly different at $P = 0.1$ according to the simulation method.
3. Each value represents the mean of 3 PDA plates per isolate. Mycelial growth was measured after 7 days and is expressed as mm/day. Means followed by the same letter/s are not significantly different at $P = 0.05$ according to Tukey's HSD test.

confirmed that these lesions were not due to the applied inocula. Isolations from symptoms classified as ray blight gave cultures of *P. ligulicola* var. *inoxydabilis* in 68% of cases. The remaining isolations either failed or had bacterial growth. None of the isolations from lesions on non-inoculated control plants yielded either *P. ligulicola* var. *inoxydabilis* or *P. exigua* var. *exigua.* For plants inoculated with the latter species, isolations yielded 61% *P. exigua* var. *exigua.*

Failed isolations again showed no mycelial growth and/or had bacterial growth. Isolations from lesions not characteristic of *P. ligulicola* var. *inoxydabilis* or *P. exigua* var. *exigua* confirmed that other fungal species were present. Fungal species found included *Alternaria* spp. (*A. alternata* and *A. tenuissima*), *Cladosporium* spp. (*C. herbarum* and *C. cladosporioides*), *Stemphylium botryosum and Ulocladium atrum.*

Spore viability varied from 32 to 99 % after 24 h. Inoculum with less than 90% germination after 24 h was checked again (another 100 conidia were counted) after 48 h and viability was always greater than 95% by this time. Isolates with low initial conidia viability were not found to have the lowest disease incidence.

CORRELATION BETWEEN LESION GROWTH AND DISEASE INCIDENCE

There was a positive correlation ($r = 0.804$, $P < 0.001$) between lesion growth in the detached leaf assay and disease incidence in the greenhouse trial for the 18 isolates screened using both techniques (Figure 6.4). Isolates with high disease incidence in the greenhouse generally had the fastest detached leaf lesion growth.

The isolates also showed a significant ($P < 0.001$) difference in mycelial growth on PDA. Mycelial growth of the *P. ligulicola* var. *inoxydabilis* ranged from 4.5 to 8.2 mm/day (Table 6.1). Five of the 6 fastest growing isolates were *P. ligulicola*

var. *ligulicola* isolates from chrysanthemum. Mean growth rates for these isolates ranged from 8.1 to 8.8 mm/day. There was a weak correlation (r = 0.391, P = 0.033) between lesion growth and mycelial growth on PDA for the 30 isolates tested.

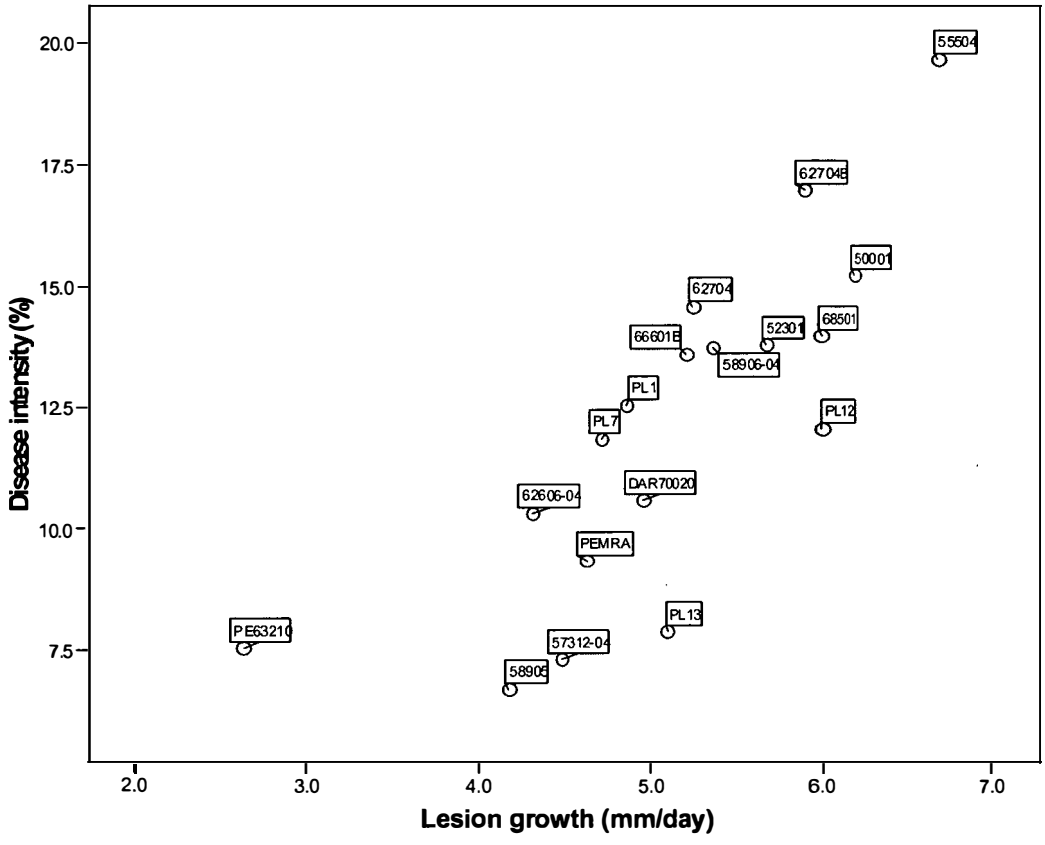

Figure 6.4. Relationship between mean lesion length in the detached leaf assay and mean disease incidence for the greenhouse experiment caused by 16 *Phoma ligulicola* var. *inoxydabilis* isolates and 2 *Phoma exigua* var. *exigua* isolates. For isolate code see Table 4.1.

DISCUSSION

This study has shown that there was variation in virulence among *P. ligulicola* var. *inoxydabilis* isolates on Tasmanian pyrethrum in greenhouse and growth chamber trials. In the greenhouse trial, disease incidence for the most virulent isolate was more than double that of the least virulent isolate (Table 6.1). Disease incidence variability was continuous, with no obvious grouping of isolates as either virulent or avirulent.

While the disease incidence of the two *P. exigua* var. *exigua* isolates was relatively low compared to the *P. ligulicola* var. *inoxydabilis* isolates, the greenhouse study confirmed the pathogenicity of *P. exigua* var. *exigua* to *T. cinerariifolium*. This is the first record of this fungus on pyrethrum. *Phoma exigua* var. *exigua* is a plurivorous fungus which has been isolated from more than 200 host genera (Aa van der *et al.* 2000) although no records were found of this fungus on pyrethrum. This fungus may be a plant pathogen associated with damping off, dieback (Abeln *et al.* 2002), bark necrosis (de Gruyter and Scheer 1998) and leaf and stem lesions, root rot or a saprophyte on dead plant material (Boerema and Howeler 1967). *Phoma exigua* var. *exigua* has been described as an opportunistic wound parasite which may also cause necrosis on stems and leaves of herbaceous plants (Aa van der *et al.* 2000). This fungal species is reported to be a weakly pathogenic or an opportunistic wound pathogen (Aa van der *et al.* 2000), however the *P. exigua* var. *exigua* isolates tested in this study were capable of infecting healthy young plants. The observed symptoms caused by *P. exigua* were not always clearly distinct from those produced by *P. ligulicola* var. *inoxydabilis.* Symptoms produced by plants inoculated with *P. exigua* were mainly leaf lesions (Figure 6.5) and occasional stem lesions, which were similar in size and colour to stem and leaf lesions caused by *P. ligulicola* var *inoxydabilis.* Inoculation with the *P. exigua* var. *exigua* isolates did not cause the characteristic pinched necrotic stem that is commonly expressed by pyrethrum infected by *P. ligulicola* var. *inoxydabilis* (Figures 6.2 and 6.3).

Figure 6.5. Lesions on pyrethrum leaf inoculated with conidia from *Phoma exigua* var. *exigua* isolate PEMRA.

In the detached leaf assay, the 30 isolates showed a continuous range of lesion growth with no aggregation of species or variety (Table 6.1). Differences in virulence were found among the Tasmanian *P. ligulicola* var. *inoxydabilis* isolates from pyrethrum and there was a two-fold difference between isolates with the fastest and slowest lesion development. As found in the greenhouse trial, there was no obvious association of geographic region or the period of storage in culture with virulence. Lesion growth was relatively slow for the two *P. exigua* var. *exigua* isolates compared to both *P. ligulicola* varieties and there was no detectable difference in lesion appearance between the two *Phoma* species. It is uncertain if the level of virulence found for the two *P. exigua* var. *exigua* isolates

is representative of the *P. exigua* var. *exigua* population on commercial pyrethrum in Tasmania because of the low number of isolates tested in this study. Additionally, changes in pathogenicity to different hosts, and increased virulence to non-preferred hosts, after successive infection and re-isolation has been reported for other fungal species (Armstrong *et al.* 1940; Chesters and Blakeman 1967). Hence further studies are required to ascertain if this phenomenon applies to *P. exigua* var. *exigua* on pyrethrum and to monitor changes in the virulence, incidence and frequency of this pathogen in Tasmanian commercial pyrethrum crops.

Lesion growth of the five *P. ligulicola* var. *ligulicola* isolates on detached pyrethrum leaves was found to be variable (Table 6.1) and not significantly different from *P. ligulicola* var. *inoxydabilis* isolates. The detached leaf assay demonstrated that the *P. ligulicola* var. *ligulicola* isolates obtained from various chrysanthemum species were pathogenic to *T. cinerariifolium*. This finding could not be further substantiated in the greenhouse study due to the possible risk of these fungal isolates being introduced into commercial pyrethrum. Inoculation of potted plants with conidial suspension, conducted in a growth chamber, is one possible way to further test the pathogenicity of *P. ligulicola* var. *ligulicola* to *T. cinerariifolium*.

The integration of information about pathogenicity and host susceptibility enables identification of host varieties that are most at risk, so that appropriate control measures can be determined. For example, the use of fungicides may be necessary on susceptible varieties but fungicide application may have little or no benefit on resistant varieties. Similarly the use of susceptible cultivars at sites considered to have a high risk of disease development can be avoided. For field grown crops, *in situ* surveys can provide a realistic account of host-pathogen-environment interactions. However field trials can be costly, labour intensive and time consuming compared to greenhouse or *in vitro* studies. Environmental and physical conditions in field trials are also harder to control or predict. Detached leaf assays have also been used to study the susceptibility

of pea (*Pisum sativum*) to *Mycosphaerella pinodes* and results of field trials were correlated (Zhang *et al.* 2006) with tests of potted plants in growth chambers (Hwang *et al.* 2006). The detached leaf assay developed and described in the current study was later used in a separate study (Pethybridge *et al.* 2008d) to assess the susceptibility of 10 pyrethrum cultivars to two *P. ligulicola* var. *inoxydabilis* isolates (isolates 55504 and 57312-04) that showed variable virulence (Table 6.1). The results generally agreed with the findings of the current study. Isolate 55504 produced faster lesion growth on all cultivars compared to isolate 57312-04. While no cultivars were found to be totally resistant to these two isolates, there were significant differences for both cultivar and isolate, and an interaction between the cultivars and isolates, indicating that some degree of vertical resistance was detected (Van der Plank 1968). Results from field trials do not always correlate well with findings from greenhouse and *in vitro* experiments (Simmonds 1991). This is because environmental variation in the field has a large impact on the expression of resistance in the field, while greenhouse and growth chamber experiments are usually carried out under a set of controlled conditions. Additionally, plant material screened in greenhouse and growth chamber tests is exposed to a limited range of pathogen genotypes. Different genotypes may occur in the field and cause a different response (Van der Plank 1982).Variation within and between greenhouse and growth chamber experiments is also inevitable due to variations in inoculum concentrations and infectivity, and variations in microenvironmenal conditions (Kong *et al.* 1997), however this type of testing provides a starting point for identification of pathotypes and disease resistant material.

For the isolates and pyrethrum variety tested here, mycelial growth did not correlate well with lesion growth. Further study may be warranted to ascertain if there are relationships between mycelial growth on PDA and lesion growth on a number of pyrethrum varieties. Physical and biological factors, and the susceptibility of different varieties, will influence lesion development.

The highly significant correlation found between the greenhouse trial and the detached leaf bioassay indicates that the latter method has potential for screening commercial pyrethrum plants for resistance to *P. ligulicola* var. *inoxydabilis* and *P. exigua* var. *exigua*. The trend was for isolates with the fastest lesion growth to produce higher disease incidence than isolates with slow lesion growth (Figure 6.4). While *P. ligulicola* favours entry through senescing or wounded tissue (Baker *et al.* 1961), mycelial hyphae and germ tubes can penetrate directly through or between epidermal walls (Baker *et al.* 1949). Wounding of plant material during the detached leaf inoculation process would have allowed the fungi to (at least partially) circumvent the infection process of penetrating the epidermis of plant tissues, which would normally take place in the absence of wounds for entry into the host. Inoculation of detached leaves with conidial suspensions or mycelial plugs may provide results that more closely relate to natural infection processes that take place in the field. Both of these methods have successfully been used to inoculate detached plant parts and potted plants of *P. ligulicola* var. *inoxydabilis* in greenhouse and *in vitro* studies (Pethybridge and Wilson 1998). A comparison of the three inoculation methods is needed to determine if the detached leaf method used in the current study can reliably be used to assess pathogenic variability of *Phoma* spp. isolates, to screen pyrethrum varieties for susceptibility and to provide results that correlate to host response and disease development in the field.

CHAPTER 7

REPRODUCTIVE MECHANISMS OF *PHOMA LIGULICOLA* ISOLATES

INTRODUCTION

Both anamorph and teleomorph have been described for the two *P. ligulicola* varieties (Aa van der *et al.* 1990; de Gruyter *et al.* 2002). However the teleomorph (*Didymella ligulicola)* has not been found on diseased plant material collected from pyrethrum crops, nor detected in *P. ligulicola* var. *inoxydabilis* cultures studied *in vitro* (Pethybridge 1998 and Ch 3 current study). Occurrence of the teleomorph is reported to be much less frequent than the anamorph (de Gruyter *et al.* 2002; McCoy *et al.* 1972). The lower incidence of the sexual stage may be partly due to more stringent environmental requirements for sexual reproduction than for asexual reproduction. Both types of reproductive structures and spores form under fluctuating temperatures but pycnidia formation occurs over a wider temperature range than perithecia production. The optimum temperature for perithecia production is 20°C and the maximum is 24°C. The optimum temperature for pycnidia production is 26°C and the maximum is 30°C (McCoy *et al.* 1972). Hence temperatures that promote formation of pycnidia may suppress perithecia development. Dry conditions promote perithecia production while moist conditions promote pycnidial development. However *P. ligulicola* is able to produce both types of reproductive structures under relative humidity ranging from 6 to 98% (Baker *et al.* 1961). Perithecia take longer to form than pycnidia. Mature pycnidia can form three days after inoculation onto chrysanthemum tissue, while production of mature perithecia take at least seven days after lesion development (McCoy 1971). While some isolates form perithecia on weak PDA when incubated at low RH, formation of the *P. ligulicola* var. *ligulicola* teleomorph on agar appears to be rare (McCoy 1971). A similar response was found in *Ascochyta rabiei* (teleomorph *Didymella rabiei*) where the teleomorph failed to develop on normal and low nutrient media in petri dishes but formed on artificially

infested chickpea straw (Trapero-Casas and Kaiser 1992). This indicates that there is a physiological response to the host or other plant material that promotes perithecia formation more readily than on artificial substrates.

Light influences formation of reproductive structures and isolates can vary in their response. On chrysanthemum *P. ligulicola* produced pycnidia and perithecia when exposed to daylight, but only pycnidia were formed when the stems were kept under constant darkness (Blakeman 1969). McCoy (1971) found an exception to this where a dark sporulating isolate formed both perithecia and pycnidia on chrysanthemum tissue under all light regimes including complete darkness. Near UV light (289-380 nm) stimulated pycnidial production in isolates that were sensitive to light, but had no influence on isolates that produced pycnidia in both light and dark conditions (McCoy *et al.* 1972). Blue and green filters placed over cultures, which were then exposed to a combination of fluorescent and incandescent lighting, stimulated perithecia production in *P. ligulicola* (McCoy *et al.* 1972). Blue light black bulbs have also induced perithecia production for various ascomycetes on water agar amended with pieces of *Hydranea* and *Gardenia* (Furukawa and Kishi 2002). Environmental races (Waggoner and Wallin 1952) of *P. ligulicola* are thought to have developed different responses to environmental stimuli through selection to historical cultural environments (McCoy and Blakeman 1976). For example, American *P. ligulicola* var. *ligulicola* isolates from field crops required UV light to produce pycnidia while European isolates from the same variety of chrysanthemum, but grown in a glasshouse, frequently produce pycnidia in total darkness (McCoy and Blakeman 1976).

Didymella ligulicola isolates from chrysanthemum were reported to be homothallic (Baker *et al.* 1949). However no specific studies have conclusively confirmed this. Various methods can be utilised to investigate the heterothallic/homothallic nature of fungi. The presence or absence of the conserved genome region known as the 'highly mobile group' (HMG), located within the mating gene, can be indicative of the fungal mating type and the heterothallic or homothallic status. Mating type studies based on PCR detection

of the HMG motif have been used for various fungi including *Cochliobolus* species (Arie *et al.* 1997), *Phaeosphaeria nodorum* (Bennett *et al.* 2003) and *Ascochyta* species (Cherif *et al.* 2006).

In vitro studies of vegetative compatibility and perithecia production can provide information on the homo-heterothallic status of fungal isolates. Homothallic isolates produce perithecia when given the right substrate and environmental conditions. Heterothallic isolates need to be paired with an isolate of the opposite mating type to produce perithecia. Mating type genes regulate the ability of nuclei to undergo karyogamy and subsequent meiosis (Coppin *et al.* 1997; Nelson 1996). When vegetatively incompatible isolates confront each other on a plate they grow out from the inoculation point until their hyphae meet and then form a clear zone known as a barrage. The barrage results from an antagonistic reaction and consists of a region of dead or dying cells between the two isolates. The mycelia either side of this region form a higher, thicker layer of hyphae and perithecia may or may not form (Leslie 1993). These laboratory based methods can be labour intensive and time consuming, especially when there are no isolates of known mating type. Mating type PCR assays provide an alternative method for determination of mating type which can complement conventional laboratory methods. There is little similarity among *MAT* genes from different genera and even among species of a single genus. However the DNA binding proteins encoded in the *MAT* genes have conserved regions which have been used to develop specific PCR primers for determination of mating type in many fungal species. Mating type PCR assays have been used for investigations of population structure, such as mating type ratios, for a range of fungi (Barve *et al.* 2003; Cherif *et al.* 2006; Rau *et al.* 2005; Zhan *et al.* 2002).

The similarity among conserved amino acids in the HMG DNA binding motif makes it a suitable as a basis for development of specific primers. The HMG has also been used as the basis for development of multiplex PCRs which detect both the alpha and HMG regions and cloning of mating type genes in ascomycetes (Arie *et al.* 1997). A PCR assay for determination of the mating

type of *P. ligulicola* isolates would provide a rapid means of determining mating type ratios in populations and provide a potential test to detect the introduction of a second mating type into an area.

The studies reported here aimed to improve our understanding of the *P. ligulicola* disease lifecycle by investigating a number of these genetic and phenotypic characteristics.

The aims of this study were to:

1. Investigate the mating system of *P. ligulicola* isolates from pyrethrum and chrysanthemum through application of a PCR-based assay.
2. To validate the findings of the PCR assay by inducing production of the teleomorph stage *in vitro.*
3. Determine whether the fungus found on pyrethrum is homothallic or heterothallic and has the capacity to develop the teleomorph stage.

MATERIALS AND METHODS

ISOLATES USED IN THIS STUDY

The five foreign *P. ligulicola* var. *ligulicola* isolates from chrysanthemum and 116 *P. ligulicola* var. *inoxydabilis* isolates assessed for biological variability in Chapter 3 (of the current study) were screened for the presence of the HMG motif in the mating gene.

DNA from single-conidium isolates was used for design of primers specific for the HMG region of the mating gene and for screening with the PCR assay. To produce single spore cultures, isolates were refreshed on PDA and incubated in darkness at 20°C for five to seven days. Each isolate was then sub-cultured onto

V8 agar (10 g agar and 100 ml V8 vegetable juice in 400 ml dH_2O, adjusted to pH 6.25 before autoclaving). Cultures were incubated under NUV light at 20 to 22°C under a day –night regime of 14 h light and 10 h darkness for 10 to 14 days. Three to four blocks of V8 agar (10 mm^3) containing pycnidia were put into 5 ml of autoclaved dH_2O in autoclaved McCartney bottles. Conidia were released from the agar by gently agitating the bottles by hand. Spore concentrations were counted with a haemocytometer and adjusted to 1×10^4 per ml water. Conidial suspension (100 µl) was spread over 4% WA using a sterile glass rod. Petri dishes were incubated in darkness at 20°C for 24 to 48 h. Individual germinated conidia were removed from WA using a sterile scalpel under 63 × magnification placed onto PDA and incubated in darkness at 20°C for 12 to 14 days. For each isolate three PDA plates were inoculated, each with a single conidium, to ensure that viable culture was recovered.

PCR ASSAY FOR DETECTION OF THE HMG IN MATING GENES

DESIGN OF HMG PRIMERS SPECIFIC FOR *PHOMA LIGULICOLA*

In collaboration with Dr Martin Chilvers and Dr Tobin Peever from Washington State University's (WSU) Pullman campus, a PCR assay to determine the mating type of *P. ligulicola* isolates was developed. DNA samples from 28 single spore Tasmanian cultures were prepared at the University of Tasmania's Cradle Coast Campus and sent to Dr Chilvers for development of HMG primers specific for *P. ligulicola.* Samples of *P. ligulicola* DNA were precipitated and dried prior to sending to Dr Martin Chilvers. A brief overview of the work done at WSU is provided in Appendix 3.

Cultures were prepared for DNA extraction, and extractions were done using the method previously described in Chapter 5 (Genetic variability).

Primers PL HMG FW2 (GGCATCAAGAAGGCACCTC) and PL HMG R236 (GCGAGTCATCCTTCCTC) (M. Chilvers, *unpublished data*) were used screen the *P. ligulicola* isolates for the HMG region. These primers were designed by M.

Chilvers and their development was based on *Ascochyta*-specific degenerate primers (HMG -L and HMG-R) (Barve *et al.* 2003). PCR reactions contained in a final volume 20 µl, 1 × Qiagen PCR buffer with 1.5 mM $MgCl_2$ (Applied Biosystems Inc., Foster City, CA), 200 µM each dNTP, 0.25 µM each primer, 0.5 units TopTaq DNA polymerase (Applied Biosystems Inc., Foster City, CA) and 20 to 40 ng DNA. PCR reactions were performed on the thermocycler described in Chapter 5. The PCR program and conditions were 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 1 min, with a final step of 72°C for 10 min. PCR products were visualised on a 1.5% agarose gel post stained with GelRed (Biotium, Hayward, CA).

PERITHECIA PRODUCTION

An *in vitro* technique for inducing perithecia production adapted from the methods of (Furukawa and Kishi 2002) was developed. A preliminary test was conducted to ascertain the most suitable method. Preliminary-test methods were based on methods used by McCoy (1971), Kaiser *et al* (1997) and Furukawa and Kishi (2002). While *P. ligulicola* is reported to produce perithecia under moist and dry conditions and fluctuating temperatures (Baker *et al.* 1949), dry conditions favour perithecia production (McCoy 1971). McCoy (1971) soaked chrysanthemum tissue in 0.5 M NaCl to reduce leaf moisture content and inoculated plant tissue with a 0.5 M NaCl conidial suspension. For *Ascochyta* spp., Kaiser (1997) soaked lentil stem pieces in conidial suspensions for 1 hour and incubated them on moistened filter paper. Furukawa and Kishi (2002) inoculated leaves of herbaceous and woody plants on WA with mycelial plugs from various fungal species including *Didymella bryoniae* and *Mycosphaerella allicina.*

Preliminary tests

Leaf treatments, inoculation methods and incubation conditions were compared in preliminary tests. One *P. ligulicola* var. *ligulicola* (NZ10673) isolate, and three *P. ligulicola* var. *inoxydabilis* (PL 7, PL12 and 62704B) were tested. Leaves were either soaked in methylated spirits for three minutes and then air dried for 10 minutes in a laminar flow; or soaked in 0.5M NaCl for 14 hours to equilibrate osmotic potential and then pressed between sheets of paper towel for two hours. Leaves were inoculated with either (i) mycelial plugs on PDA, (ii) with conidial suspensions in dH_2O containing approximately 10^4 spores /ml or (iii) with conidial suspensions in 0.5M NaCl containing approximately 10^4 spores /ml. Leaves were soaked in conidial suspensions for 30 min and then drained for 10 min. Isolates were tested alone and in paired combinations for mycelia plugs and conidial suspensions. Each leaf-inoculation treatment was incubated in a petri dish alone, a petri dish with a moistened filter paper or a petri dish containing 4% water agar. Each leaf-inoculation-plate treatment was done twice for each isolate. Inoculated leaves were incubated in a growth chamber at 20°C under 4 blue light black bulbs (model L36W/73d, Osram, Munich) with a 14 h light and 10 h darkness regime. Leaves were examined three times a week for production of pycnidia and perithecia.

PERITHECIA PRODUCTION ON PYRETHRUM LEAF AMENDED AGAR

Petri plates with 4% WA were amended with pyrethrum leaves from six month old pyrethrum cultivar 'A' plants grown in a greenhouse. Leaves were soaked in methylated spirits for three minutes and left to air dry for 10 minutes in a laminar flow before being placed onto the water agar surface. Leaves were inoculated with mycelial plugs taken from actively growing fungal cultures. The plates were sealed with gladwrap incubated in a growth chamber at 20°C under 4 blue light black bulbs (model L36W/73d, Osram, Munich) (14 hours light/10 hours darkness).

Leaves were examined for development of pycnidia or perithecia three times per week. Fruiting structures were removed from infected leaves, stained with aniline blue and examined at 400 × magnification. When ascospores were detected on the slides, individual ascospores were liberated by moistening leaf surfaces with distilled water. To ensure there was no runoff or drip, a small volume of water (~200µl per leaf) was applied to each leaf with a pipette. Plates with moistened leaves were inverted over another petri dish containing 4% water agar. The two plates were sealed with cling-wrap and incubated at 20°C in darkness for 12 hours. Individual germinated ascospores were then isolated from the bottom dish and placed onto potato dextrose agar to create single ascospore cultures. These cultures were incubated in darkness at 20°C. After 7 to 10 days mycelial plugs were taken from the single-ascospore cultures, placed onto pyrethrum leaf amended water agar, incubated in a growth chamber at 20°C under blue light black bulbs and monitored twice a week for production of pycnidia and perithecia. Additional leaf amended WA plates were incubated on a bench near a window at ambient room temperature for the five *P. ligulicola* var. *ligulicola* isolates and four *P. ligulicola* var. *inoxydabilis* (55503, 55504, PP15 and PL19). The five *P. ligulicola* var. *ligulicola* isolates were tested individually and the *P. ligulicola* var. *inoxydabilis* isolates were tested individually and in pairs.

VEGETATIVE COMPATIBILITY TESTS

Vegetative compatibility was tested for 12 Tasmanian *P. ligulicola* var. *inoxydabilis* isolates and five foreign *P. ligulicola* var. *ligulicola* isolates. All possible pairs of isolates were tested twice on both V8 and PDA agar. Each agar plate was inoculated with three or four 5mm mycelial plugs, taken from three isolates. Mycelial plugs were placed approximately 20mm from other plugs. Each isolate were tested for self-compatibility by placing two mycelial plugs from the same culture onto the agar. All isolates were refreshed on PDA and grown in darkness at 20°C for 7 to 10 days prior to testing for vegetative

compatibility. PDA plates were incubated in darkness at 20°C and V8 agar plates were incubated at 20°C under an 18W cool daylight fluorescent globe (TL-D18W/1865, Phillips) light with a 14 h light period and 10 h darkness regime. Plates were checked for vegetative compatibility, barrage formation and development of pycnidia and perithecia for three weeks after inoculation.

NUCLEI STAINING

Because pseudohomothallic fungi can have conidium with two nuclei of each mating type (Nelson 1996) a giemsa staining technique was adapted from Shirane *et al.* (1988) to determine the number of nuclei in conidia produced by two Tasmanian and three foreign *P. ligulicola* isolates. Fungal cultures were grown on V8 agar and incubated under flourescent light at 20°C for 14 days. Conidia were collected from the isolates by placing several 1 cm blocks of agar into distilled water. Conidia were washed three times in distilled water and the resulting suspension was adjusted to a concentration of 1×10^8 spores/ml. Glass slides were pre-coated with a thin layer of egg white and conidial suspensions were mixed into the egg white (one isolate per slide). The coated slide was soaked in fixer (3:1 methylated spirits : glacial acetic acid) for 30 minutes and then washed with 95% ethanol. Slides were then soaked in 70% ethanol overnight, removed and immersed in 5 M HCl for 30 min. After washing with dH_2O, slides were immersed in freshly prepared giemsa stain (10 ml giesmsa stock in 150 ml phosphate buffer pH 7.0) for three hours. Slides were air dried for 15 minutes before observation under oil immersion at 1000 × magnification. The number of nuclei in 200 randomly selected conidia was determined for each of the five isolates.

RESULTS

Single conidium cultures were produced for all isolates. For three *P. ligulicola* var. *inoxydabilis* isolates, an extreme culture type with very little aerial mycelium and copious pycnidia was produced in one of the three cultures (Figure 7.1). These extreme culture types have been found in several fungal species after single-spore culturing but often require several successive single-spore series of 20 cultures to occur (Hansen 1938). The three *P. ligulicola* var. *inoxydabilis* isolates produced pycnidia in complete darkness and the entire culture was covered with pycnidia with conidia indistinguishable from *P. ligulicola.* These culture types differed to the remaining single conidium cultures, and to the isolates described in Chapter 3 which were either the mycelia type, with few or no pycnidia, or an intermediate type with moderate aerial mycelium and moderate pycnidia/ conidia production.

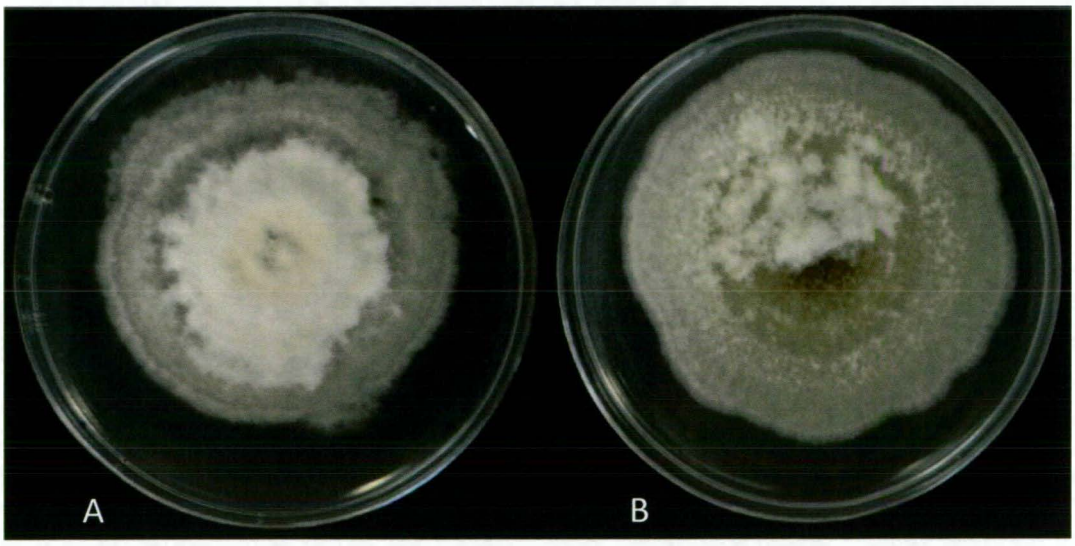

Figure 7.1. Original culture of isolate 62704B on PDA (A) and dark sporulating single spore isolate from 62704B (B). The original culture (A) did not produce pycnidia when grown in complete darkness. The dark sporulating isolate (B) has been refreshed from -80°C storage for one year and has reverted to *MC* type with aerial mycelium but has retained the capacity to sporulate in complete darkness.

PCR DETECTION OF THE HMG IN MATING GENES OF *PHOMA LIGULICOLA*

One hundred and twenty three *P. ligulicola* isolates were screened using the PL-HMG specific primers. A 220 bp region of the MAT HMG was detected in all five *P. ligulicola* var. *ligulicola* isolates sourced from chrysanthemum (ATCC 10748, DSMZ 63133, DSMZ 62547, ICMP 2287 and ICMP 10673) and in one Tasmanian *P. ligulicola* var. *inoxydabilis* isolate from pyrethrum, isolate 55503(Figure 7.2). The HMG motif was not detected in the remaining 115 Tasmanian isolates.

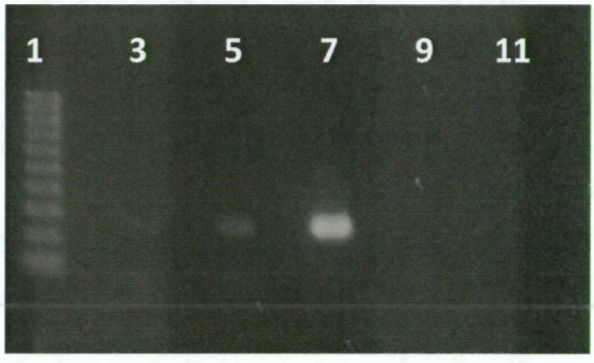

Figure 7.2. Amplification of the 220 bp HMG region with specific primers PL HMG FW2 and PL HMG R236 (M. Chilvers, *unpublished data*). Lanes: 1, 100 bp ladder; 3 and 5, Tasmanian *Phoma ligulicola* var. *inoxydabilis* isolate 55503 from pyrethrum; 7, New Zealand *Phoma ligulicola* var. *ligulicola* isolate ICMP 10673 from chrysanthemum; 9 Tasmanian *Phoma ligulicola* var. *inoxydabilis* isolate PL1 from pyrethrum, 11 no DNA added to PCR reaction. Two separate DNA samples of *Phoma ligulicola* var. *inoxydabilis* isolate 55503 were tested and both had mild positive PCR results.

PERITHECIA PRODUCTION ASSAY

Preliminary test results

There was little or no mycelial growth and no production of reproductive structures for the isolates on leaves in petri dishes only or with filter paper. With the exception of leaves inoculated with suspensions of conidia in 0.5M NaCl, all isolate/inoculation combinations produced mycelia and pycnidia on leaves incubated on water agar. *Phoma ligulicola* var. *ligulicola* isolate

DSMZ 63133 produced pycnidia and mature perithecia with ascospores when grown alone on leaf amended water agar, for both leaf treatments. Production of pycnidia and perithecia was faster on leaves treated with methylated spirits (12 and 20 days respectively) than on leaves treated with NaCl (20 and 42 days). *Phoma ligulicola* var. *inoxydabilis* isolates produced pycnidia and conidia within 7 to10 days. Perithecia were not detected on leaves inoculated with single or pairs of *P. ligulicola* var. *inoxydabilis* isolates, and no ascospores were found. These isolates were monitored for 10 weeks because ascomycetes can take up to eight weeks to develop reproductive structures on diseased plant material (Kaiser *et al.* 1997).

PERITHECIA PRODUCTION ON PYRETHRUM LEAF AMENDED AGAR

Five foreign *P. ligulicola* var. *ligulicola* from chrysanthemum and 10 Tasmanian *P. ligulicola* var. *inoxydabilis* isolates were tested in the perithecia production assay. The five *P. ligulicola* var. *ligulicola* were initially tested alone because of the positive HMG PCR result, and because isolate DSMZ63133 produced perithecia in the preliminary tests. The *P. ligulicola* var. *inoxydabilis* isolates were tested individually (one isolate per leaf amended agar plate (Figure 7.3)) and in pairs to test for compatibility. Isolate 55503, the only *P. ligulicola* var. *inoxydabilis* that was positive for the HMG motif, was included.

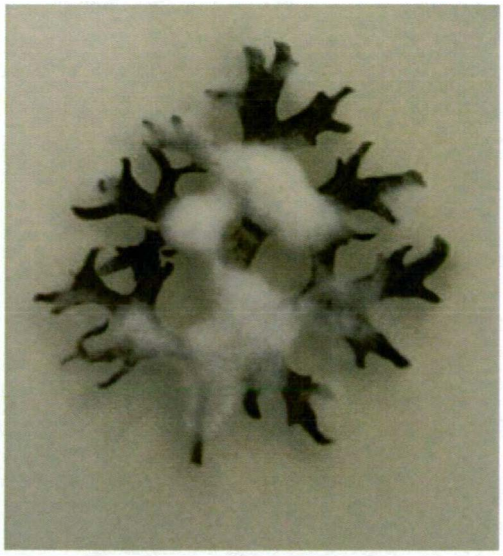

Figure 7.3 Aerial mycelium on pyrethrum leaf 5 days after inoculation with *Phoma ligulicola* var. *inoxydabilis* isolate PL4 from pyrethrum in Tasmania.

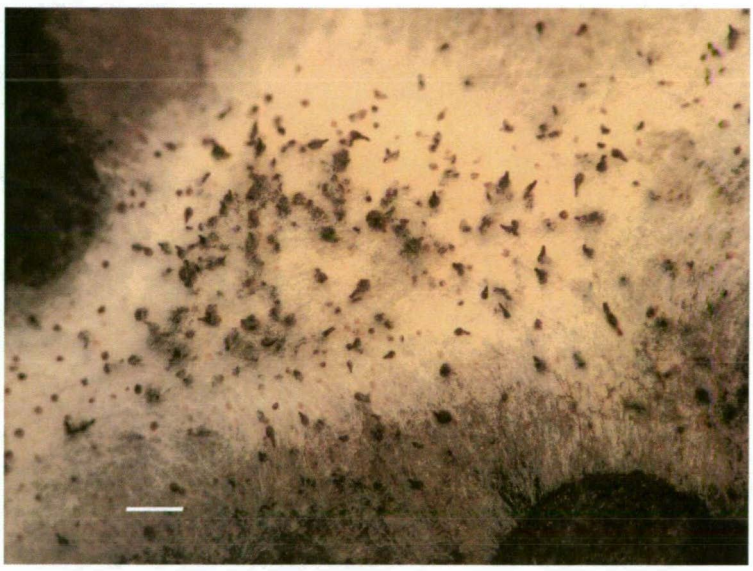

Figure 7.4 Aerial mycelium and reproductive structures of *Phoma ligulicola* var. *ligulicola* isolate DSMZ 62457 (from chrysanthemum in Germany) on pyrethrum leaf amended 4% WA. Larger dark flask shaped structures are perithecia and smaller globose saffron coloured structures are pycnidia. Bar = 1 mm.

Four of the five foreign isolates (DSMZ 63133, DSMZ 62547, ICMP 2287 and ICMP 10673) produced ascospores on their own and did not require pairing with other isolates. Perithecia containing asci of various maturity and mature ascospores were produced three to four weeks after inoculation under both light regimes (BLB and natural/fluorescent light) (Figures 7.4 and 7.5). Perithecia abundance varied from relatively dense patches to rare and many perithecia were sterile (no ascospores). The single ascospore cultures derived from these perithecia also produced perithecia and ascospores after 21 to 28 days. No perithecia or ascospores were detected for the remaining foreign isolate (ATCC 10748) from the USA or for the 10 Tasmanian isolates.

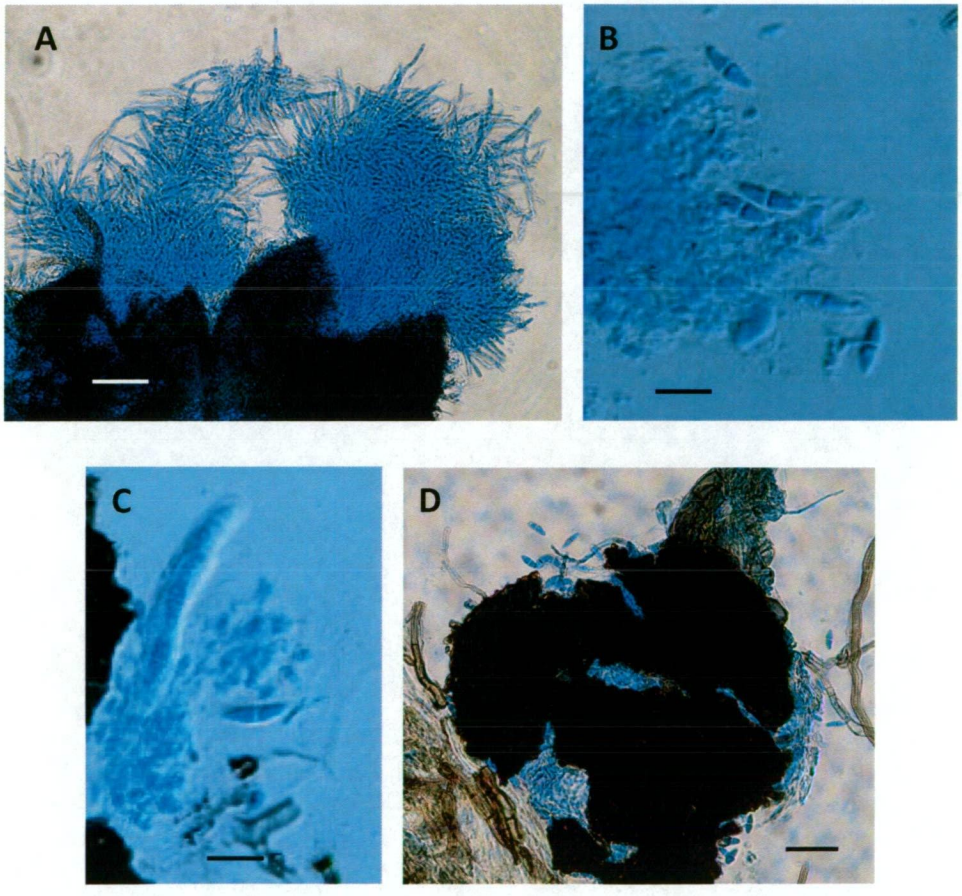

Figure 7.5 (A) Perithecia from *Phoma ligulicola* var. *ligulicola* isolate DSMZ 62457 with asci at various stages of maturity. *Phoma ligulicola* var. *ligulicola* isolate ICMP 10673 (B) ascospores, (C) ascus and ascospore and (D) perithecium and ascospores. Bars = (A) 50 µm (B-C) 12 µm; (D) 50 µm.

VEGETATIVE COMPATIBILITY TESTS

All isolates were vegetatively incompatible with other isolates and themselves. All isolate pairings formed a distinct barrage zone (Figure 7.6). All isolates formed pycnidia which were distributed over the colony (in various quantities) and not concentrated at the barrage. *Phoma ligulicola* var. *ligulicola* from Germany and New Zealand produced sterile perithecia, as well as pycnidia, on V8 agar.

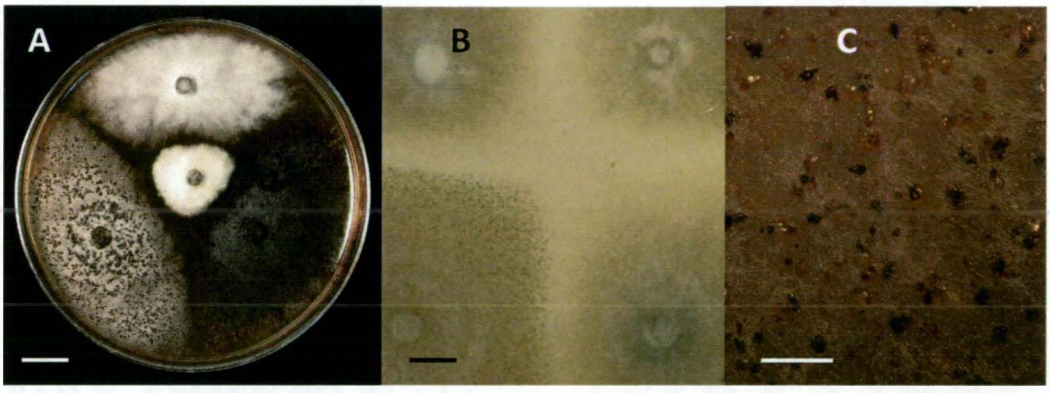

Figure 7.6 Vegetative compatibility tests of *Phoma ligulicola* isolates (A) PL4 (centre) ATCC 10748 (top), DSMZ 62547 (left) and DSMZ 63133 (right) on PDA; (B) *Phoma ligulicola* var. *inoxydabilis* PL4 (top left and right) and 55503 (bottom left) and 55504 (bottom right). Barrage zones of inhibited mycelial growth are visible between the all isolates. (C) Sterile perithecia and pycnidia produced by *Phoma ligulicola* var. *ligulicola* isolates DSMZ 62547 on V8 agar when grown alone. Bar = (A) 10 mm; (B) 5 mm; (C) 1 mm.

NUCLEI STAINING

The five isolates had mainly uninucleate spores but also produced binucleate spores (Table 7.1; Figure 7.7). Spores containing more than two nuclei were not detected.

Table 7.1. The percentage of *Phoma ligulicola* conidia with one or two nuclei. Two hundred conidia were assessed for each isolate.

Isolate	Variety	Origin	Uninucleate conidia (%)	Binucleate conidia
ATCC 10748	*ligulicola*	USA	94	6
DZSM 62547	*ligulicola*	Germany	93	7
ICMP 2287	*ligulicola*	New Zealand	97	3
PL12	*inoxydabilis*	Wynyard, Tasmania	97	3
PL13	*inoxydabilis*	Sassafrass, Tasmania	95	5

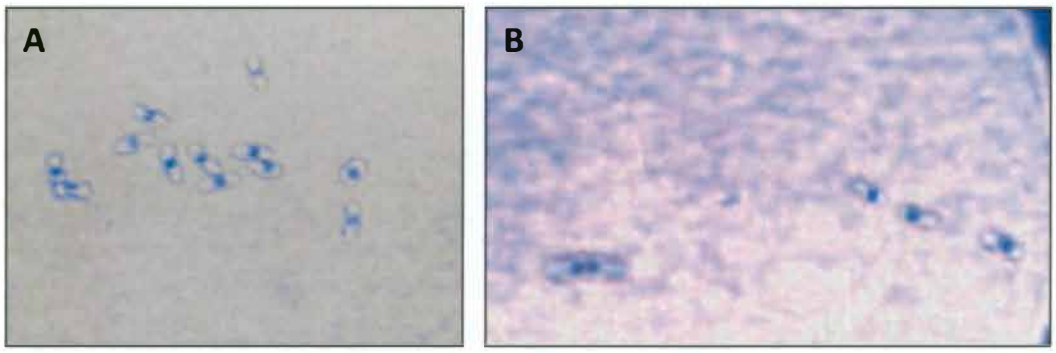

Figure 7.7. Giemsa stained nuclei of conidia of *P. ligulicola* var. *ligulicola* isolate DSMZ 62547. (A) Uninucleate conidia; (B) One binucleate spore (lower left) and three uninucleate spores (right).

The formation of perithecia without pairing with other isolates, and the positive result for HMG indicates that the isolates *P. ligulicola* var. *ligulicola* are homothallic. These findings agree with the assumption that *P. ligulicola* is homothallic by Baker *et al.* (1949) and with more recent studies where perithecia production occurred *in vitro* on chrysanthemum plant tissue with no reported 'pairing' or mating of isolates (McCoy *et al.* 1972). There was no reference to varieties of *P. ligulicola* in either of these reports, but the two varieties were not differentiated (by the NaOH test) until 1990 (Aa van der *et al.* 1990). Isolates used in the studies of Baker *et al.* (1949) and McCoy (1972) were sourced from chrysanthemum and are therefore assumed to be var. *ligulicola.*

Isolate ATCC 10748 was the only *P. ligulicola* var. *ligulicola* that failed to produce perithecia on pyrethrum amended water agar. Pycnidia production was also rare for this isolate (Chapter 3 current study). This isolate may have lost the ability to produce perithecia due to long-term storage and repeated subculturing on agar media. *Pleospora herbarum* isolates lost the ability to produce perithecia when they were subcultured more than three times on PDA but retained perithecia production when subcultured four times on leaf amended agar (Furukawa and Kishi 2002). Alternatively, the environmental parameters for perithecia production may be different or more stringent for this isolate than for the other four *P. ligulicola* var. *ligulicola* isolates. Similar variation has also been reported for other fungi. Isolates of *Ascochyta rabiei* from Iran, Turkey, India and West Pakistan failed to produce the teleomorph (*Mycosphaerella rabiei* Kovacevski) on infected chickpea while isolates from Bulgaria and Greece formed the sexual stage (Kaiser 1973). This may indicate that isolate ATCC10748 may have different light, temperature or nutrient requirements. Another possible explanation is that this isolate is host specific and requires inoculation on chrysanthemum to produce the sexual stage. Most reports of *P. ligulicola* var. *ligulicola* perithecia production are on inoculated chrysanthemum and not on other hosts or agar, regardless of the incubation

condition (Aa van der *et al.* 1990; Baker *et al.* 1949; 1961; de Gruyter *et al.* 2002; McCoy 1971). Further studies of isolate-host interaction are needed to ascertain if host specificity is a requirement for perithecia production in isolate ATCC 10748.

By comparison, *Phoma ligulicola* var. *inoxydabilis* is reported to commonly produce perithecia on *in vitro* cultures (Aa van der *et al.* 1990; Boerema *et al.* 2004; de Gruyter *et al.* 2002). However, the teleomorph has not been detected for Tasmanian isolates in culture or on pyrethrum. The apparent absence of the teleomorph in pyrethrum crops, and the failure of the isolates from Tasmanian pyrethrum to form perithecia *in vitro*, could be due to genotypic adaptation. The Tasmanian isolates may have developed different reproductive mechanisms to the isolates studied in published accounts through selection and adaptation to different hosts and environmental conditions. Adaptation to a specific host can result in a high level of diversity among pathogen populations (Leung *et al.* 1993). Homothallic and heterothallic species often occur within closely related genera of ascomycetes. The genus *Neurospora* contains heterothallic, pseudohomothallic and homothallic species (Metzenberg and Glass 1990). The *Phytophthora* genus contains both homothallic and heterothallic species (Moore and Frazer 2002) and strains of self-fertile homothallic species that apparently have only one mating type (Nelson 1996). Hence it is plausible that the two *P. ligulicola* varieties differ in their mating systems. The negative results of the HMG specific PCR assay, for all but one of the Tasmanian *P. ligulicola* var. *inoxydabilis* isolates, suggests that they are heterothallic and that the majority of the isolates are the alpha mating type (*MAT-1*). There is also a possibility that negative results for the HMG PCR for most of the *P. ligulicola* var. *inoxydabilis* isolates are due to divergence in the mating gene region for these isolates. A PCR which can detect either the HMG or the alpha region of the mating gene, and preferably both, for the Tasmanian *P. ligulicola* isolates is needed to confirm the initial findings and support the claim that they are heterothallic. Detection of the alpha region for the foreign isolates that were positive for the HMG is also needed to validate the homothallic status.

The Tasmanian *P. ligulicola* isolates were sampled for determination of mating type distribution on two geographic scales, regional and within field. The prevalence of *MAT-1* isolates is consistent with asexual reproduction and indicates that both populations are likely to be mainly clonal. This is not surprising for the within-field samples. Greater mating type diversity for the between-field isolates was expected because they were sampled from a wide geographic range over a three year period. Mating type ratios that differ significantly from 1:1 limit the chance of sexual reproduction (Barve *et al.* 2003). The low incidence of *MAT-2* mating types found for *P. ligulicola* var. *inoxydabis* means that there is little opportunity for sexual reproduction. However, in the absence of the sexual recombination, this pathogen can still adapt to changing environments through selection and successive mutations (Milgroom 1996; Van der Plank 1982).

Isolate 55503 was the only Tasmanian *P. ligulicola* var. *inoxydabilis* isolate that tested positive for the HMG and is presumed to be *MAT-2.* Pairing of this *MAT-2* isolate with several designated *MAT-1* isolates failed to induce perithecia production under any of the conditions used in this study (pairing on PDA, V8 agar and on pyrethrum leaf amended WA). Barrage zones formed between the two cultures for all confrontations of isolate 55503 and other *P. ligulicola* var. *inoxydabilis* isolates designated *MAT-1.* Barrage formation does not exclude sexual reproduction and formation of perithecia (Leslie 1993). However no perithecia were detected for any isolate combinations. The reasons for this are not clear. Isolate 55503 may have lost the ability to produce the sexual stage. This isolate did not produce pycnidia on OA (Chapter 3, current study) and produced only very few pycnidia on V8 agar. Because of this, it was also not possible to determine the number of nuclei in conidia produced by this isolate.

The extreme culture type produced for three isolates from only one series of single-spore culturing is of interest. This occurrence, called the 'dual phenomenon' has been found in many imperfect fungi including *Ascoshyta pisi*, *Sphaeropsis* spp. and *Phoma* spp. (Hansen 1938). An intermediate culture type (*MC*) is thought to be the most common type and is made up of the two extreme

culture types: one characterised with mycelia and few or no pycnidia or conidia (*C*), and the other which produces profuse pycnidia and only a small amount of mycelia (*M*). Subsequent single-spore culturing of *M* and *C* types produce only *M* or *C* cultures however they produce *MC* types when mixed together in culture. All three culture types (*M*, *C* and *MC*) appear in a single series of single-spore cultures for *Phoma terrestris*, which has binucleate spores. Since Hansen, the dual phenomenon has been observed in many fungi including *Trichophyton mentagrophytes* (Robin) Blanchard and *Epidermophyton floccosum* (Harz) Langeron and Milochevitch (Wilhelm 1947), strains of *Aspergillus niger* van Tieghem (Rai and Tewari 1961) and various *Fusarium* species (Nelson *et al.* 1983). The dual phenomenom is thought to be due to genetically different nuclei within a single hyphae or conidium (Hansen 1938). This segregation into extreme types has not been found in teleomorphs, which are uninucleate, and is not believed to be connected to mating type. However it may compensate for the lack of sexual reproduction ability and provide some genetic variability for asexual fungi (Hansen 1938). While *M* type cultures of *Phoma ligulicola* var. *inoxydabilis* have been isolated from diseased pyrethrum crops, the majority are *MC* and the *C* type has not been isolated. This indicates that the former two types are most common, and that the sterility found in some freshly obtained isolates from pyrethrum fields is 'normal' and not through loss of ability to sporulate while in culture. Variability in sporulation capacity and apparent sterility has also been found for fresh *P. ligulicola* isolates from chrysanthemum fields (Baker *et al.* 1949; 1961).

Binucleate conidia were observed in isolates of *P. ligulicola* var. *ligulicola* and *P. ligulicola* var. *inoxydabilis* (Table 7.1, Figure 7.6). The finding of binucleate conidia in the two *P. ligulicola* var. *inoxydabilis* isolates raises the possibility of pseudohomothallism in the apparently heterothallic Tasmanian isolates. Pseudohomothallic filamentous ascomycetes are self fertile because they contain two nuclei of each mating type in a single spore (Nelson 1996). The absence of the teleomorph in culture and the low number of binucleate spores suggest that pseudohomothallic behaviour is either very rare or not occurring in the Tasmanian *P. ligulicola* isolates. Similarly, the three *P. ligulicola* var.

ligulicola that were tested had up to 7% binucleate conidia and could potentially also be pseudohomothallic. Again this seems unlikely because cultures derived from single liberated ascospores produced perithecia for four of the five isolates.

The number of nuclei within individual conidia, and the size (volume) of conidia increased with increased concentrations of glucose for the pseudohomothallic fungus *Botrytis cinerea* (teleomorph *Botryotinia fuckeliana*) (Phillips *et al.* 1987). Temperature influences conidium size of *P. ligulicola* and lower temperatures promote larger conidia (McCoy *et al.* 1972). Further studies are needed to ascertain if the influence of temperature on conidial size observed in *P. ligulicola* by McCoy translates into differences in nuclei number and subsequent pseudohomothallism in *P. ligulicola* var. *inoxydabilis*. Additionally, determination of nuclei number for *P. ligulicola* var. *inoxydabilis* 55503, the only isolate from pyrethrum found to be positive for the HMG PCR is needed.

The failure of the *P. ligulicola* var. *inoxydabilis* to form perithecia and the PCR results indicate that they are heterothallic. Conditions that promoted perithecia production for four of the five *P. ligulicola* var. *ligulicola* isolates did not promote perithecia formation in any Tasmanian *P. ligulicola* var. *inoxydabilis* isolates, either singly or in pairs. The two *P. ligulicola* varieties appear to have different mating systems and possibly require different environmental conditions to develop the sexual stage. If the Tasmanian *P. ligulicola* population is heterothallic and more than one mating type is present in close proximity, sexual reproduction of *P. ligulicola* could occur and result in genetically divergent progeny. The resultant progeny could possess characteristics which enhance pathogen fitness and the ability to survive in the Tasmanian environment. The ratio of mating types found in this study indicates that the chances of sexual reproduction are low. If the sexual stage is neither present, nor likely to develop (e.g. due to unfavourable environmental conditions, geographic separation of mating types or inadequate mating type ratios), an important source of aerially dispersed primary inoculum is absent from the ray blight disease cycle on pyrethrum in Tasmania.

CHAPTER 8

GENERAL DISCUSSION

This study of biological variability of *Phoma* species associated with pyrethrum and chrysanthemum considered fungal isolates as distinct entities for measurement of morphological, physiological and genetic traits. The information gained through this study represents baseline information that will enable monitoring of population changes over time.

Morphological and cultural variability of *P. ligulicola* isolates was characterised in this study. The findings generally agreed with published accounts, which also report cultural variability among isolates. However some discrepancies were found. The morphological criteria examined in this study did not reliably differentiate the two *P. ligulicola* varieties. Several isolates of *P. ligulicola* var. *inoxydabilis* had characteristics reported to occur only in *P. ligulicola* var. *ligulicola* including zonation and faster mycelial growth on malt extract agar (MA) than on oatmeal agar (OA). Another discrepancy with published accounts of the *in vitro* behaviour of this fungus was the failure of *P. ligulicola* var. *inoxydabilis* to produce perithecia in culture. The NaOH test was the only characteristic that clearly differentiated the two varieties. This method requires two weeks of incubation but is relatively inexpensive and simple. The detached stem bioassay showed that isolates of *P. ligulicola* var. *ligulicola* obtained from chrysanthemum were able to infect *T. cinerariifolium.* Additionally, this variety infected leaves and produced conidia and perithecia in the mating type studies. Hence it is important to monitor all new isolations from fields to ascertain if this variety has found its way from domestic chrysanthemum plants into commercial pyrethrum crops.

Similarly, the EF1- α and G3PD sequences did not separate the two *P. ligulicola* varieties. In both gene regions, *P. ligulicola* var. *ligulicola* isolates from Germany

were identical to Tasmanian *P. ligulicola* var. *inoxydabilis* isolates from pyrethrum. The ITS was the only genetic region that consistently differentiated the two varieties. However there was not enough variability in the ITS region for development of specific PCR primers for either variety.

While the Tasmanian population appears to be largely clonal, comparison of the ITS and G3PD trees with the EF1- α indicates that genetic recombination has occurred. Genetic regions vary in their rate of change and evolution, and different regions may have different histories of descent (Milgroom 1996). The test for congruence among the trees for the ITS, G3PD and EF1-α regions found conflict among the sequence data partitions. Incongruence between trees based on different genes is indicative of genetic recombination (Guttman and Dykhuizen 1994; Milgroom 1996). Hence the differences found among the three gene regions indicates that genetic recombination occurred in each of the *P. ligulicola* varieties, and that some regions have remained constant within the two varieties while others have evolved. Of the three regions assessed, the EF1- α phylogenetic tree was most similar to groupings from AFLP analysis (Jason Scott *pers. comm.*). Both analyses grouped *P. ligulicola* var. *inoxydabilis* isolate PL4 with the American *P. ligulicola* var. *ligulicola* ATCC 10748, and placed the German isolates closer to the Tasmanian isolates than the New Zealand isolates. Further studies are needed to find if other DNA based methods, such as restriction enzyme digest, can distinguish between the two varieties.

Conidia produced on OA are the primary morphological feature used to confirm the identity of *Phoma* species (Boerema *et al.* 2004). Approximately 15% of the *P. ligulicola* var. *inoxydabilis* isolates used in the current study did not sporulate on OA making conidial measurement and examination impossible and conclusive species identification difficult. Variability in sporulation of *P. ligulicola* from chrysanthemum, even in fresh isolates, has been reported (Baker *et al.* 1949; 1961). Production of conidia was more reliable on V8 agar, and this may provide an alternative for isolates that do not produce conidia on OA. However, comparisons between the two media need to be made because media

type influences conidial morphology. The inability of isolates of *P. ligulicola* var. *inoxydabilis* to form the teleomorph *in vitro* also meant that ascospores, an important morphological feature, could not be observed in this study. This highlights the variable nature of this fungus and the difficulty of identifying *Phoma* spp. based only on morphology and cultural characteristics. It also indicates that DNA based methods play an important and complementary role to traditional methods used in fungal population studies.

This phylogenetic study clarified the identity of several *P. ligulicola* isolates that were previously ambiguous due to cultural variability and the inability to examine conidia. The findings of this study also suggest that a high proportion of the *P. ligulicola* var. *inoxydabilis* population is clonal. This is supported by the absence of the teleomorph, *Didymella ligulicola*, in Tasmania which also implies that there is less potential for genetic recombination of *P. ligulicola* var. *inoxydabilis* found on pyrethrum. However, adaptation and evolution of the asexual stage is still likely to occur because selection pressure will increase or decrease certain genotypes and phenotypes. Pathogen populations that reproduce asexually may contain physiologically separated, genetically identical, individuals (clones) which behave and adapt differently (phenotypes). Through selection pressure, groups of individuals (genotypes and phenotypes) then evolve over time and become better adapted to their environment (Russel 1994). The importance of sexual reproduction to the epidemiology of *P. ligulicola* is not clear. In terms of disease management for ray blight of pyrethrum, the lack of sexual reproduction means that an important source of airborne inoculum is absent from the Tasmanian pyrethrum industry.

The findings of the HMG PCR and the *in vitro* perithecia production experiment indicate that the foreign isolates from chrysanthemum are homothallic, and the Tasmanian isolates from pyrethrum are heterothallic. *Phoma ligulicola* var. *ligulicola* isolates from chrysanthemum in New Zealand and Germany produced the teleomorph with no pairing and were positive for HMG. The isolate from America was also positive for the HMG PCR but did not produce perithecia or

ascospores. This isolate may have lost the ability to produce ascospores or may have different requirements for sexual reproduction. A PCR assay to detect the alpha region is needed to conclusively validate the homothallic status of these isolates, but could not be developed within the time constraints of this study. Detection of the alpha region is also needed to confirm the heterothallic status of the *P. ligulicola* isolates from pyrethrum. The HMG region was detected in only one of 116 Tasmanian isolates, indicating that there are two mating types and one mating type (*MAT-1*) is predominant. Pairing of the *MAT-2* isolate with several *MAT-1* isolates did not yield perithecia under conditions that were conducive for teleomorph formation in *P. ligulicola* var. *ligulicola* isolates. Inducement of the teleomorph, either *in vitro* or *in vivo*, under experimental conditions is needed for confirmation of the heterothallic status indicated by the mating type PCR. Further work is required to determine if the environmental parameters for teleomorph formation differ for the two *P. ligulicola* varieties. Development of a more conclusive PCR based *MAT* assay which allows detection of both the alpha and HMG regions for identification of mating type is also needed.

Self fertilisation is common for homothallic fungi and if there is no outcrossing, a strongly clonal population will result. However, outcrossing does occur for some homothallic fungal species (Anderson and Kohn 1995). For example, strains of two homothallic species, *Sordania fimicola* and *Emericella nidulans*, were found to cross readily in the laboratory (Fincham *et al.* 1979). Future studies could investigate the potential of the (homothallic) *P. ligulicola* var. *ligulicola* isolates to outcross with each other and with the (heterothallic) *P. ligulicola* var. *inoxydabilis* from pyrethrum. If successful crosses can be made, the resultant progeny could be assessed for their morphology and pathogenicity to pyrethrum.

The methods used in this study to assess pathogenic variability have potential to supplement information collected from field trials and for screening pyrethrum cultivars for susceptibility. The detached stem assay and the

greenhouse trials characterised virulence and detected differences among isolates of *P. ligulicola* var. *inoxydabilis.* Breeding disease resistant plant material is a desirable disease control strategy which has the potential to reduce input costs associated with other forms of disease management (for example fungicide application) for the pyrethrum industry. The information gained in this study provided baseline information and will allow selection of isolates with variable virulence to screen pyrethrum plant material for susceptibility to *P. ligulicola* and *P. exigua.* The pathogenicity assessment methods used in this study also have potential for studies that examine environmental factors that influence disease development in this pathosystem. Determination of these environmental parameters would increase our understanding of the pathogen-host relationships. This information could also facilitate development of a predictive model for disease forecasting specifically targeting ray blight on pyrethrum in the Tasmanian environment and provide a decision management tool for the pyrethrum industry.

This is the first study to characterise the baseline sensitivity to difenoconazole in *P. ligulicola* var. *inoxydabilis* and *P. exigua* populations obtained from pyrethrum in Australia (Jones *et al.* 2007). This sensitivity profile will provide valuable information for tracking future resistance development of this pathogen to members of the DMI fungicide group. The two *P. ligulicola* var. *inoxydabilis* populations from pyrethrum contained isolates that were generally sensitive to the DMI fungicide difenoconazole. The sensitivity distributions for these two populations were continuous, which indicates that resistance development for this fungicide-pathogen system is a slow and directional process. Because shifts in DMI fungicide response are generally gradual and occur over lengthy periods making frequent checks on sensitivity will not necessarily be beneficial for monitoring resistance (Brent 1991). However ongoing monitoring of the current disease control methods is advisable to ensure sustainable fungicide effectiveness. The pyrethrum industry monitors practical performance of fungicides in the field by measuring the amount of disease in field experiments (Pethybridge *et al.* 2007a; Pethybridge *et al.* 2005a;

Pethybridge *et al.* 2009; Pethybridge *et al.* 2008b; Pethybridge *et al.* 2008d; Pethybridge *et al.* 2008f; Pethybridge *et al.* 2006b; 2007b). Observations by field officers and growers can also reveal changes in response to a fungicide if they are aware of the symptoms of this disease. If deterioration in disease control in the field is detected visually, *P. ligulicola* isolates can be collected and tested for sensitivity. These results can then be compared to the sensitivity data generated in the current study, to see if resistance is the cause. Other probable causes for disease control failure, such as poor fungicide application or unusual weather conditions can be ruled out or investigated, depending on the result of the new sensitivity data.

This is the first report of *P. exigua* var. *exigua* on pyrethrum. A combination of morphological and genetic characterisation was used to identify this fungus. While some morphological criteria overlapped (mycelial growth and conidial dimensions) for the two *Phoma* species, all three gene regions in this study separated the four *P. exigua* isolates from *P. ligulicola* isolates. Pathogenicity tests confirmed that this fungus can cause disease of pyrethrum. The fungicide sensitivity assay showed that the *P. exigua* isolates were not highly sensitive to the DMI, difenoconazole. Due to the small number of isolates assessed in this study it is not possible to determine if these isolates are representative of the larger *P. exigua* population, or if they are extreme types. However the reduced sensitivity is of concern for the pyrethrum industry as selection pressure may increase the prevalence of this pathogen. Additional isolates need to be collected and tested to determine this. The low isolation frequency of *P. exigua* compared to *P. ligulicola* from field samples indicates that the former fungus may not be as well adapted to survive and cause disease on pyrethrum. The lower lesion growth and disease incidence shown by the two *P. exigua* isolates supports this theory. Again, the small number of isolates tested makes it difficult to determine if they are representative of the broader *P. exigua* population. Further studies are needed to ascertain if *P. exigua* var. *exigua* is pathogenic and fungicide resistant enough to be a serious problem for the pyrethrum industry.

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Appendix 1

Protocol: Isolation of Total DNA from fungal tissue using the DNeasy Plant Mini Kit (50) # 69104 (Qiagen, GmbH, D40724, Hilden Germany) (Chapters 4 and 7)

1. Weigh out 100 mg of mycelium for each sample.
2. Grind samples in liquid nitrogen with mortar and pestle and place in autoclaved microcentrifuge tube.
3. Immediately after grinding, add 400 µl Buffer AP1 (Lysis buffer) and 4 µl RNAase A solution.
4. Vortex vigorously and then incubate for 10 minutes in water bath at 65°C.
5. Add 130 µl Buffer AP2 (Precipitate buffer - supplied with kit), mix and incubate on ice for 5 min.
6. Centrifuge at 13000 rcf for 5 min.
7. Remove supernatant and add to shredder column (supplied with kit).
8. Centrifuge shredder column at 13 000 rcf for 2 min.
9. Transfer flow through liquid to a new tube without disturbing the cell-debris pellet.
10. Add 1.5 volumes (~675 µl) of Buffer AP3/E (Binding buffer- supplied with kit) to the lysate and mix by pipetting.
11. Apply 650 µl of the mixture from previous step to a white spin column (supplied with kit) and centrifuge at 8000 rcf for 1 min.
12. Discard flow though and repeat previous step with remaining sample mixture. Discard flow-though and collection tube.
13. Place DNeasy Mini Spin Column in a new 2 ml collection tube (supplied in kit). Add 500 µl Buffer AW (Wash Buffer) to the spin column and centrifuge at 13000 rcf for 1 min.
14. Discard flow-through and re-use the same collection tube. Add 500 µl Buffer AW (Wash Buffer) to the spin column and centrifuge at 13000 rcf for 2 min to dry the membrane.
15. Transfer the DNeasy Mini Spin Column to a new 1.5 or 2 ml microcentrifuge tube and pipette 100 µl of preheated (65°C) Buffer AE (Elution buffer) onto the DNeasy Mini Spin Column membrane.
16. Incubate at room temperature (15 to 25°C) and then centrifuge for 1 min at 8000 rcf.

Appendix 2.

Ethanol precipitation post-sequencing reaction clean-up protocol (Chapter 4).

The procedure outlined below follows instructions based on Beckman Coulter instructions, with slight modifications as recommended by Roger Latham, (Research Assistant, Australian Food Safety Centre of Excellence, University of Tasmania, Sandy Bay Campus).

See Beckman Coulter instruction sheet (P/N 608118) supplied with Dye Terminator Cycle Sequencing (DTCS) Quick Start Kit (P/N 608120) and list of reagents below.

Procedure

1. Make up the Stop Solution/ Glycogen mixture (2 µl 3M Sodium Acetate (pH 5.2), 2 µl 100mM Na_2-EDTA (pH 8.0) and 0.3 µl Glycogen (supplied with kit) per sample. Make mixture in microcentrifuge tube immediately before use working at room temperature.
2. Vortex mixture after adding glycogen.
3. Label one microcentrifuge tube per reaction. Label sides and top as the ethanol may remove labelling.
4. Aliquot 4.3 µl Stop Solution/Glycogen mixture into each of the labelled microcentrifuge tubes.
5. Working on ice, transfer the sequencing reaction to the appropriately labelled microcentrifuge tube and mix thoroughly.
6. Working on iced, add 60 µl 95% ethanol (v/v) from -20°C freezer and mix thoroughly. Immediately centrifuge tubes at 14, 000 rpm for 30 minutes at 4°C. **The maximum speed of our centrifuge was only 13,200 rpm.*
7. Remove supernatant with pipette taking care not to disturb the pellet.
8. Rinse the pellet with 200µl 70% ethanol (v/v) from -20°C freezer by running the ethanol down the opposite side of the tube to where the pellet is located. Do not mix or pipette up and down as this will disturb the pellet.
9. Centrifuge immediately at 13,200 rpm for 5 minutes at 4°C.
10. Repeat steps 7 and 8.
11. Remove ethanol again, without disturbing the pellet and vacuum dry for 10 minutes or leave tubes open in dark laminar flow cabinet until pellets are dry (1 – 2 hours).
12. Add 30µl of sample loading solution (supplied with DTCS kit) to dried tubes and mix well by pipetting 20 times.
13. Store tubes in -20°C freezer until loading into sequencing equipment.

Appendix 3.

Summary of work done with Dr Martin Chilvers at Washington State University for HMG PCR assay (Chapter 7)

The following work was done at the Washington State University's plant pathology laboratory during August 2006. DNA samples from 28 single spore Tasmanian cultures were prepared at the University of Tasmania's Cradle Coast Campus and sent to the USA prior to my arrival. Work conducted during my visit to the WSU Pullman Campus included the following:

Initial screening of 12 *P. ligulicola* isolates (used as positive controls) for the presence of HMG motif using two degenerate primer sets of varying degeneracy in a PCR with a temperature gradient to determine the most suitable annealing temperature. The most degenerate primers (HMG -L and HMG-R) (Barve *et al.* 2003) amplified a 220 bp product. Cloning was deemed necessary to make further progress in sequencing the HMG domain for the *P. ligulicola* isolates.

Cloning of isolate DSMZ 63133 was done using a QIAGEN PCR Cloning Dit (Qiagen Cat 231222/231224). Briefly the procedure involved mixing the PCR product with Qiagen pDrive Cloning Vector and Ligation Master Mix, followed by incubation at 16°C for 30 min. Two microliters of the ligation-reaction mixture was added to a tube of QIAGEN EZ Competent Cells, then incubated on ice for 5 min. The mixture was then subjected to heat shock by immersion in a water bath at 42°C for 30 s followed by two minutes on ice. Next, 250 µl SOC medium (Qiagen) was added to the reaction mixture. The transformation mixture was then plated directly onto three Luria Bertani (LB) agar plates containing ampicillin. To enable blue/white screening we applied 50µl X-gal (20 mg/µl) and 10 µl IPTG (100mM) onto the LB agar plates and allowed it to dry before spreading the transformation mixture over the plates. These plates were then incubated at 37°C for 15 hrs in an inverted position. A number of either

blue or white colonies formed on the LB plates during this incubation period. White colonies have the desired DNA insert while the blue colonies do not.

Small quantities of DNA from 20 single white recombinant colonies were used as template for a PCR reaction using HMG-L and HMG-R primer set. These same 20 colonies were also subcultured into 5ml LB broth + 1 µl/ml ampicillin and incubated at 37°C (on an angled shaker) overnight, and then stored in 100% glycerol (as back-up material for later use if required). The PCR of the plasmid DNA produced a ~4100 bp product (~3850 bp Cloning Vector plus ~270 bp insert). The plasmid DNA from one of the 20 clones was then extracted using QIAprep Miniprep kit (Cat #27104/27106) and sequenced using the Big Dye reaction and the HMG-L and HMG-R primers. Edge Biosystems - Performa Gel Filtration Cartridges were used to prepare the reactions for Big Dye sequencing. All sequencing was performed in the Laboratory for Biotechnology and Bioanalysis, School of Molecular Biosciences, WSU, Pullman.

Sequences resulting from the clone were aligned using Invitogen NTI software and specific primers were designed (PL HMG FW2 and Pl-HMG-R236). These primers were used in a PCR to screen *P. ligulicola* isolates for the presence of the HMG motif.